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BIOCHEMICAL EFFECTS OF SIMULATED AIR POLLUTANTS

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31 December 1962

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AP-182

(Formerly RG-6999)

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Frances L. Estes

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JAN. 1, 1960 – DEC 31, 1962

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GALVESTON, TEXAS

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Summary

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Galveston, Texas

BIOCHEMICAL EFFECTS OF SIMULATED AIR POLLUTANTS

First Progress Report
January 1, 1960 - December 31, 1962

Further examination of the inhibition of the growth of E. Coli by the air pollution mixtures has indicated that the activity of aldolase and of glutamic dehydrogenase is also inhibited and pointed to the oxidation of sulfhydryl groups as one of the sites of reactivity of the pollutant mixture.

Attempts to fractionate or concentrate the biologically effective material has served to indicate that biological effectiveness did not reside in a single component and implicated polar material(s).

Since the effect of the pollutant mixture on succinoxidase system was comparable to that of 2-nitropropane the latter may serve as a model compound for the examination of effects on particular enzyme systems.

The improved control in the redesigned apparatus for producing the pollutant mixtures provided for consistancy and reproducibility in the chemical analyses and thus should permit more definative comparisons of chemical analyses and biological effects. Under conditions in which ozone was not formed, the disappearance of NO_2 and the unsaturated hydrocarbon reactants pointed to product(s) from interaction of these materials as the biologically effective agent(s).

December 31, 1962

Part II

A. Production of Simulated Air Pollutants

For the production of simulated oxidant type air pollutant mixture, hydrocarbons, nitrogen oxides and air were irradiated with ultra-violet light in an all glass flowing system. (Gast, Schwartz and Estes, Am. Chem. Soc. 132nd Meeting, Abstr. 36M, New York City, 1957). On the input side nitrogen oxides were supplied by bubbling air through concentrated nitric acid at 50 ml/min. The hydrocarbons were intermittantly injected and the reactants carried into a heated pre-mix flask by a stream of compressed air, total flow 400 cc/min. Heat from the ultra-violet lamps maintained the irradiated flasks at 75-85° C. A sketch of the apparatus has been shown in Figure 1, which indicates the division of flow for an unirradiated control line and also includes one quadrant of the ultra-violet lamp assembly surrounding the reaction flasks.

Attempts to set up a continuous hydrocarbon feed have been unsuccessful. At the flow rates in question, around 5 µl/hour, even with a capillary orifice evaporation of the lower individual hydrocarbons and "gumming" with a hydrocarbon mixture (straight run gasoline) occurred. Thus injection of the hydrocarbons has continued to be intermittant. Although the resulting variations in the reaction have not been apparent in the usual chemical analysis of the effluent, when samples were taken at varying times after the hydrocarbon injection a variable pattern for gas chromatographic analysis was observed. When the injection was 2.5 µl/30 min., samples taken at about 5-15 minutes after injection showed a developing peak for the hydrocarbon which disappeared from samples taken 15-20 minutes after injection. This suggested a cyclic pattern of reactions was occurring which varied with the concentration of the hydrocarbon. The variation in

the chromatograph peak became less marked when smaller amounts of the hydrocarbon were injected more frequently (1.25 μl/15 min.). In Table I a comparison of the usual chemical analysis does not indicate this difference.

The analytical procedures routinely used included the phenolphthalin method for oxidants (Lacoq, Bull. Soc. Chem. Belg. 54, 186, 1945), and Saltzman's method for nitrogen dioxide (Anal. Chem. 26, 1949, 1954). Acidity was determined by a back titration of the standard alkali solution. Ultra-violet absorbing substances were trapped in 4% sodium hydroxide, scanned from 400 to 220 mμ on a Beckman DK-2 and arbitrarily expressed as nitrate.

Exploration of ultra-violet absorbing compounds (Gast and Estes, Anal. Chem. 32, 1712, 1960) indicates that in the presence of a base, aliphatic nitro compounds absorb more strongly in the 300 mμ region than does the nitrate ion. Comparisons of the input of nitrogen oxides with the effluent, indicated that the observed NO_2 and NO_3 exceeded the total input of nitrogen oxides. In part this may be accounted for by the observation that the phenol disulfonic acid method does not account for all of the combined nitrogen oxides since it cannot account for the total calculated input from the nitric acid. These observations, and the development of a method for determining aliphatic nitro compounds (Estes and Baughman, Anal. Chem. 33, 473, 1961), led to examination of the effluent for the presence of aliphatic nitro compounds. Unfortunately, the method for recovery and determination is limited, especially for nitromethane and nitroethane, by the extractants. Hence the observation that aliphatic nitro compounds were not present in the effluent may reflect the lower limit of the method rather than the absence of these materials.

Preliminary studies on the variation of the pollutant mixture with reactants has been shown in Table II. From the chemical analysis it is evident that nitrogen oxides are required for the formation of oxidant as well as the unidentified 302 mμ absorbing material. Although oxidant formation required both nitrogen oxides and hydrocarbon it appears that the branch chain hydrocarbons lead to higher oxidant values. This does not imply that the oxidant materials are necessarily the same substance. The need for extensive repairs on our gas chromatographic equipment precluded comparison of these analyses with the chromatographic patterns.

The above described system for preparing the pollutant mixture was used in the investigations included in sections B, C, F, G, and H of this report. Attempts to compare the chemical analyses and biological effects (appendix B) prompted the redesign of the apparatus for the production of the pollutant mixture. The design, evaluation and studies with this apparatus have been appended (appendix A) to make clear that it was not the system used in the biological studies included in the body of the report.

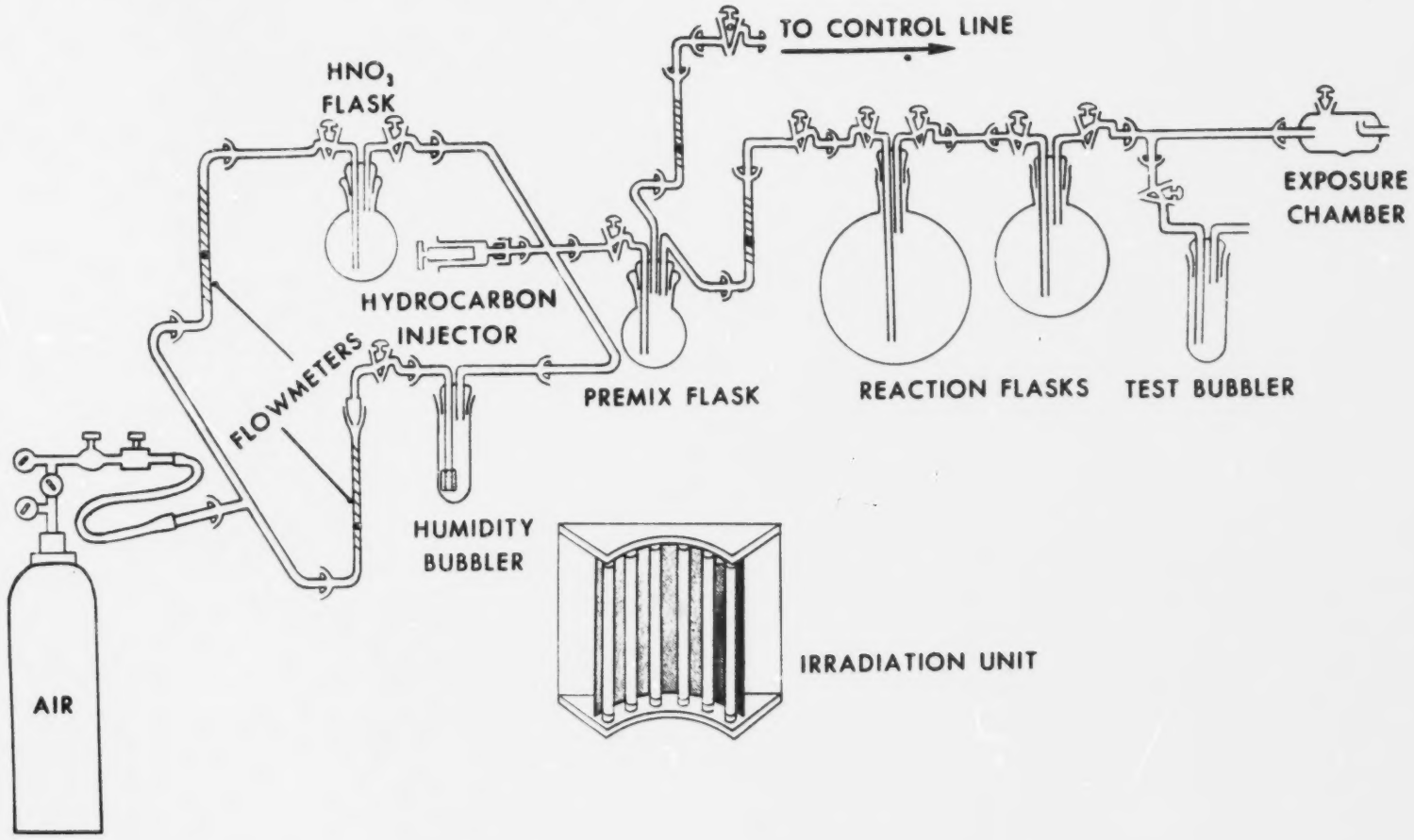
TO CONTROL LINE
HNO_3 FLASK
EXPOSURE CHAMBER
HYDROCARBON INJECTOR
FLOWMETERS
PREMIX FLASK
REACTION FLASKS
TEST BUBBLER
HUMIDITY BUBBLER
IRRADIATION UNIT
AIR

Figure 1

Table I

EFFECT OF TIME BETWEEN INJECTIONS OF MIXED HYDROCARBONS

OXIDANT ($\mu g\ O_3/l$)	NITROGEN DIOXIDE (ppm NO_2)	ACID (meq/l)	302 mμ ABSORBING MATERIAL (mg NO_3/l)
Injection of 2.5 ul at 30 minute intervals:			
5.9-10.0	0.39-▶2.92	0.015-0.022	2.34-2.66
5.5-6.4	0.38-0.41	0.015	2.46-2.70
5.2-6.6	0.35-0.39	0.018-0.018	2.70-2.70
6.0-6.6	0.32-0.41	0.017-0.018	3.09
5.0-5.9	0.28-0.39	0.018-0.021	2.21-2.83
6.9-7.3	0.22-0.33	0.020-0.024	2.70-2.83
5.9-5.9	0.33-0.21	0.019	1.99-2.57
6.6-7.1	0.05-0.21	0.019-0.020	2.21-2.34
Injection of 1.25 ul at 15 minute intervals:			
2.1-3.7	0.21	0.019-0.021	2.46-3.48
2.1-2.7	0.33-0.38	0.023-0.024	1.10-3.35
4.7-4.8	0.22-0.37	----	2.70-2.70
4.8-6.2	0.32-0.53	0.024-0.026	2.46-2.70
4.1-6.0	0.41-0.56	0.032-0.033	2.40-3.02
5.5-7.1	0.27-0.38	0.023-0.026	2.40-3.02
6.0-7.3	0.33-0.38	----	2.90-3.35
6.2-7.1	0.22-0.37	----	2.57-2.96
6.6-16.6	0.41-▶2.92	----	3.22-3.76
6.4-9.2	0.27-1.23	----	2.10-3.63

EXPERIMENTAL CONDITIONS:

50 cc air/minute bubbled through HNO_3.
Total air flow 400 cc/minute.
Flow rate through test solutions 200 cc/minute.
Irradiated at 75-85° C.

Values given as a range over 2-3 hour period.

Table II

VARIATION OF REACTANTS

REACTANTS ADDED TO AIR	PRODUCTS*			
	OXIDANT ug O_3	NITROGEN DIOXIDE ppm NO_2	ACID meq	302 mμ ABSORBING MATERIAL mg NO_3
None	<0.1-0.1	0.01-0.03	0.002-0.002	0.00-0.00
N-oxides	1.0-1.2	0.80-0.83	0.020-0.020	1.64-1.75
N-oxides	0.0-1.0	0.88-0.83	0.017-0.018	1.75-1.86
N-oxides	0.9-1.1	0.61-0.78	0.015-0.016	----
N-heptane	0.6-0.6	0.04-0.04	0.000-0.001	0.09-0.09
N-heptane	0.3-0.4	0.00-0.01	0.001-0.001	0.00-0.20
N-heptane	0.0-0.1	0.01-0.01	0.001-0.001	0.20
N-heptane	0.0-0.1	0.00-0.00	0.000-0.000	0.09-0.15
Iso-octane	0.2-0.2	0.01-0.01	0.001-0.002	0.00-0.00
2-octene	0.0-0.1	0.01-0.01	0.001-0.001	0.04-0.09
Mixed hydrocarbon	0.0-0.2	0.01-0.01	0.001-0.001	0.20-0.20
N-oxides, N-heptane	3.7-4.3	0.16-0.16	0.018-0.018	1.86-1.86
N-oxides, N-heptane	5.0-5.2	1.54-1.57	0.020-0.021	1.64-1.75
N-oxides, N-heptane	3.9-4.1	0.12-0.16	0.016-0.019	1.86-1.86
N-oxides, Iso-octane	7.8-14.1	>2.92->2.92	0.022-0.023	1.75-1.75
N-oxides, Iso-octane	4.7-4.8	0.16-0.19	0.017-0.018	1.64-1.75
N-oxides, Iso-octane	3.7-3.7	0.16-0.17	----	----
N-oxides, Iso-octane	3.9-3.9	0.11-0.41	0.016-0.019	----
N-oxides, Iso-octane	3.9-3.9	----	----	----
N-oxides, mixed hydrocarbon	5.5-6.4	0.38-0.41	0.015	2.46-2.70
N-oxides, mixed hydrocarbon	5.2-6.6	0.35-0.39	0.018-0.018	2.70-2.70
N-oxides, mixed hydrocarbon	5.9-5.9	0.33-0.21	0.019	1.99-2.57

EXPERIMENTAL CONDITIONS:

For N-oxides, 50 cc air/minute bubbled through HNO_3.
Total air flow 400 cc/minute.
Flow rate through test solutions 200 cc/minute.
2.5 ul hydrocarbon injected at 30 minute intervals.
Irradiated at 75-85° C.

*Values given as a range over 2-3 hour period.

B. Effects of the Pollutant Mixture on Growth of Escherichia coli*

Following the observations of Goetz and Clark on the killing effect on Escherichia coli of aerosols produced by irradiation of auto exhaust, studies were initiated to adapt the millipore filter technique to our studies of the pollutant mixture on growth of these organisms. After apparatus was devised for an exposure chamber and a growth chamber the technique proved to be both sensitive and reproducible (Pan, Gast and Estes, Applied Microbiology 9, 45-51, 1960). The technique requires exposing a thin layer of organisms collected on millipore filter paper to the gaseous agent, transferring the bacteria to the growth chamber and determining the growth lag by following the change in optical density of the medium.

The growth lag following exposure of E. coli to the synthetic oxidant type pollutant mixture has been reproduced in Figure 2. (Ibid.). The growth of the organisms was followed by change in optical density as shown on the abcissa. The growing time after exposure and transfer to fresh media was shown on the ordinate. For the 15 minutes exposure the organisms were not killed but were definitely inhibited.

In order to establish that no single input component of the pollutant mixture was responsible for this inhibition, the organisms were exposed to mixtures of air-heptane; air-2-octene; and air plus mixed hydrocarbons. After two hours exposure (7 hours incubation) there was no observable difference between the exposed organisms and the unexposed control. The effect of exposure to air-nitrogen oxide mixture has been shown in Figure 3.

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*Presented at the Symposium on Air Pollution (Division of Water and Waste Chemistry) American Chemical Society 138th Meeting, New York City, September, 1960.

After 90 and 120 minute exposure to the nitrogen oxides alone some inhibition was observed but less than with the synthetic pollutant mixture.

Growth is a complex phenomenon, the sum of a number of reactions. Hence the inhibition of growth per se, was not particularly indicative of the reaction or reactants involved. However, a reversal of this inhibition by particular materials could be indicative of the reactions involved in the organisms.

Preliminary survey of the effects of additives to the growth medium is shown in Figure 4. When either yeast extract or a casein hydrolysate was added to the medium the rate of growth was comparable to that of the unexposed control.

These observations indicated that the effects of individual amino acids should be examined. Although several amino acids reversed the inhibition after varying lag times, the addition of glutamic and aspartic acid to the growth medium gave consistant and definite reversal of the inhibition. For a more critical test of the reversal longer exposure times (2 hours) were used so that the growth of the exposed organisms in the unsupplemented medium was inhibited for more than 18 hours.

In Figure 5 has been shown a comparison of the effects of glutamic and aspartic acids with their α-keto counterparts. The effect of fumaric acid is not shown in the figure since it was identical to that of α-ketoglutaric acid. After 18 hours the negative control was still completely inhibited. The exposed organisms in which the growth medium had been supplemented with α-ketoglutaric acid and with fumaric acid were also completely inhibited at the end of 18 hours.

These experiments suggested that the carbon chain was not involved and that either amination and/or transamination was involved in the

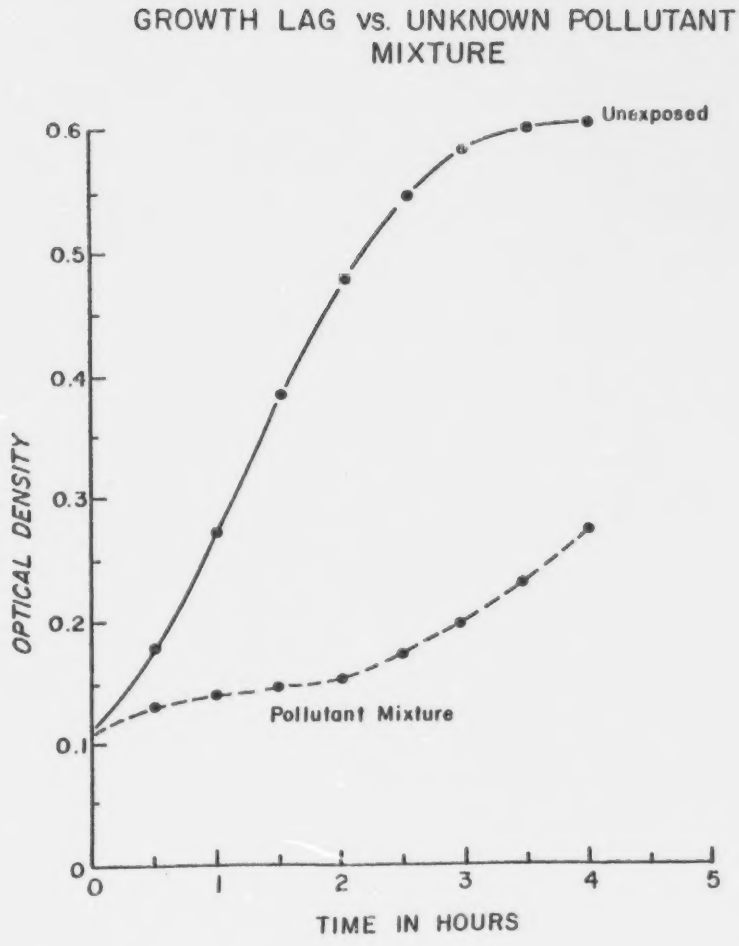
GROWTH LAG vs. UNKNOWN POLLUTANT MIXTURE
Unexposed
Pollutant Mixture
OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
TIME IN HOURS

Figure 2

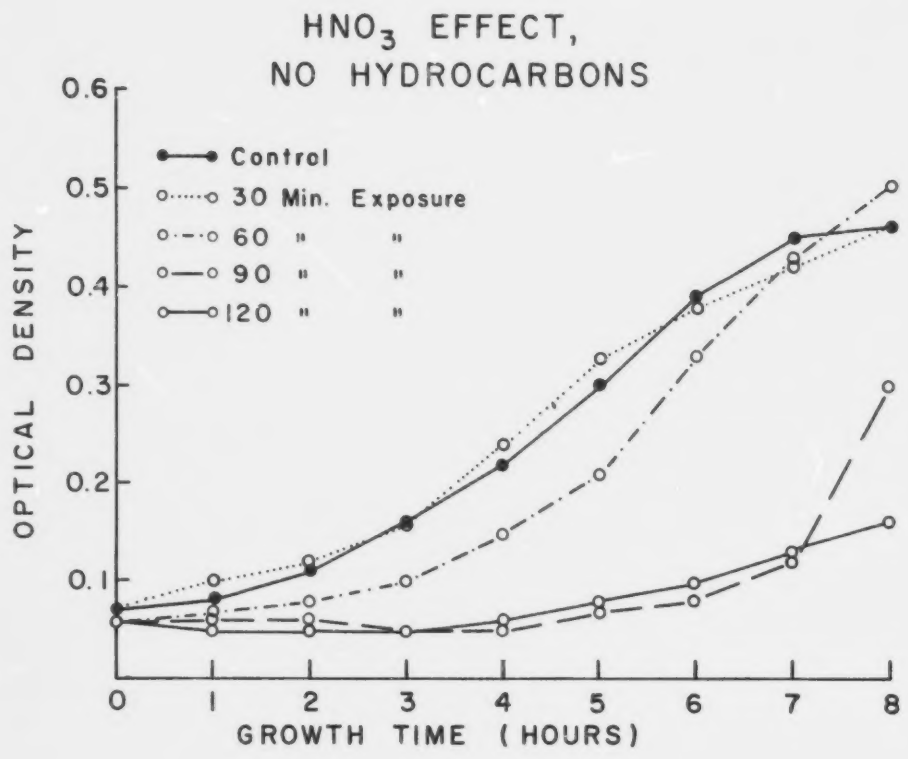
HNO3 EFFECT,
NO HYDROCARBONS
Control
30 Min. Exposure
60 " "
90 " "
120 " "
OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
8
GROWTH TIME (HOURS)

Figure 3

inhibition. These reactions and their coenzymes are:

1) alanine + α-ketoglutarate $\underset{\text{pyridoxyl-phosphate}}{\overset{\text{transaminase}}{\rightleftharpoons}}$ pyruvate and glutamate

2) DPNH + NH_4^+ + α-ketoglutarate $\underset{\text{dehydrogenase}}{\overset{\text{glutamic}}{\rightleftharpoons}}$ glutamate and DPN

The transaminase requires pyridoxyl-phosphate as coenzyme and another amino acid as a source of the amino group. This reaction is reversible and serves in the formation of other amino acids from glutamate which in turn can be formed from the amination, reaction 2.

In the growth medium for E. coli, ammonium salts served as the sole source of nitrogen. Thus if the transaminase reaction alone was involved, glutamate would still be formed from reaction 2, and the formation of other amino acids would be inhibited such that higher concentrations of glutamate were required.

In a few experiments amino acids other than glutamic and aspartic acids also gave a reversal of the inhibition. One such experiment is shown in Figure 6. This observation suggested that the transaminase reaction required exploration. Initial experiments pointed to pyridoxyl--phosphate, the coenzyme, as a possible site of inhibition. Repeated experiments, however, failed to confirm this observation. Age and concentration of the organisms, exposure times and the pyridoxyl-phosphate solution were carefully controlled. These completely opposed results have not yet been explained. Examination of the possibility that they reflect variation in concentration of materials in the pollutant mixture was precluded by a variety of difficulties which prevented complete analysis of the pollutant mixture for the particular exposures in question.

In Figure 7 has been shown a typical experiment in which pyridoxyl--phosphate alone did not reverse the inhibition. Although a combination

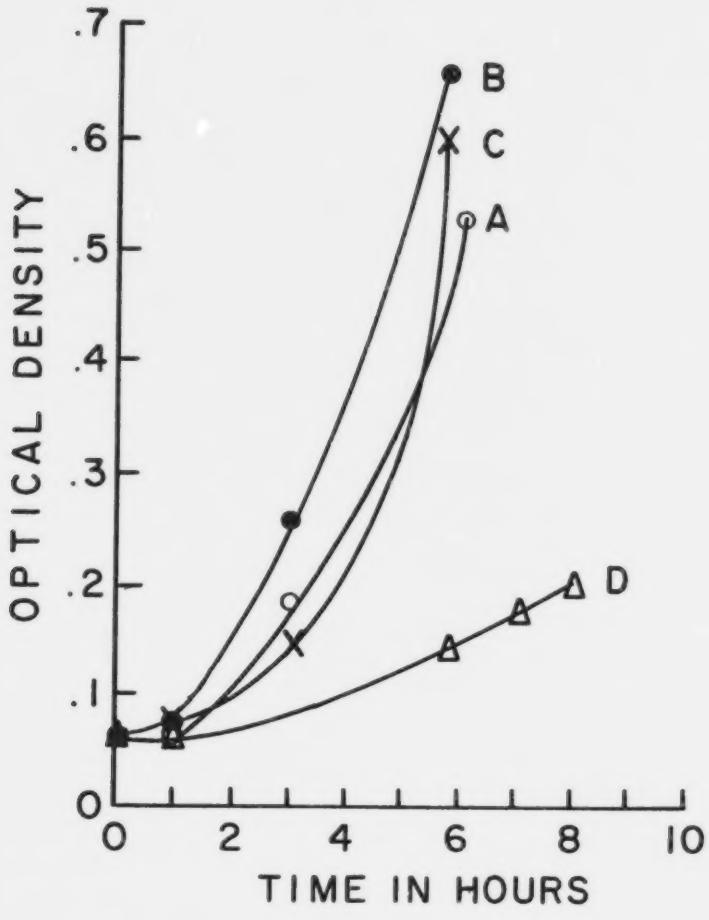

Figure 4. Effect of additives on growth of E. coli exposed to an oxidant type pollutant mixture. Exposure time 30 minutes, oxidant concentration 5.6 μg O_3/1.
Curve A exposed to pollutant mixture, casamino acid (Difco) added to 0.1% concentration.
Curve B exposed to pollutant mixture, yeast extract (Difco) added to 0.1% concentration.
Curve C exposed to air, without additive (control).
Curve D exposed to pollutant mixture, without additive.

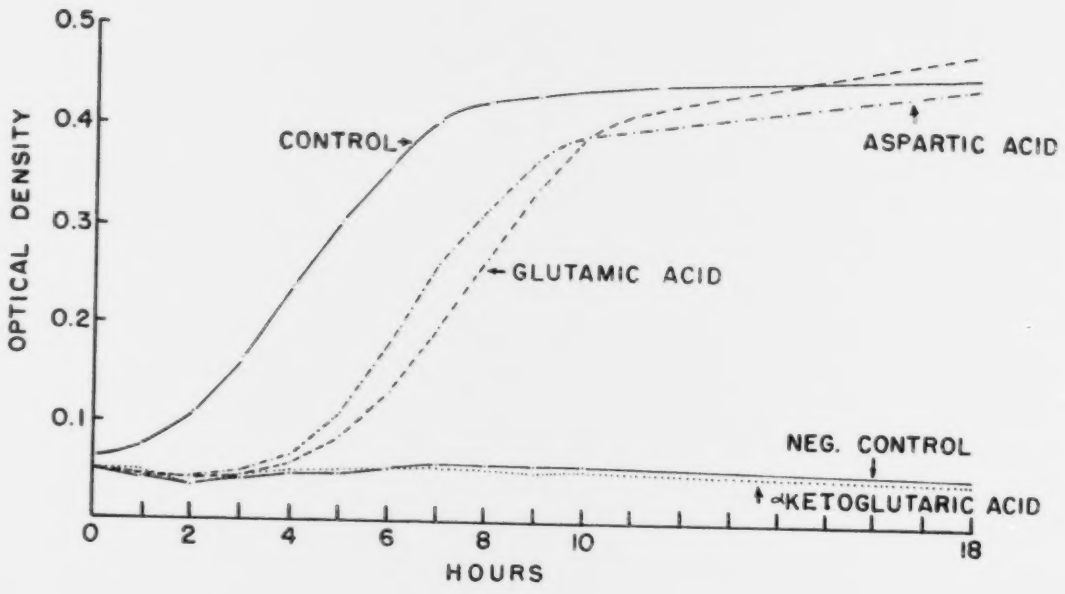

Figure 5. Effect of added glutamic acid and aspartic acid on the growth of E. coli following exposure to simulated oxidant type air pollutants. Exposure time 120 minutes.
Incubated in C-medium (inorganic medium containing NH_4Cl as source of nitrogen and added glucose as energy source) with 0.5 mmoles of glutamic acid, aspartic acid and α-ketoglutaric acid added as indicated.

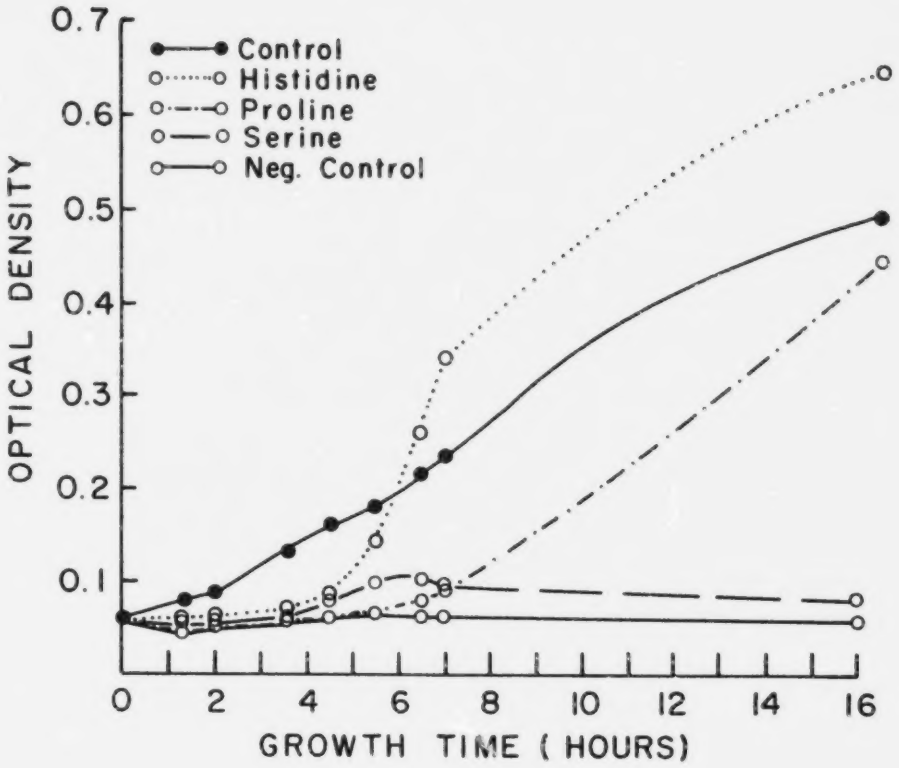
Control
Histidine
Proline
Serine
Neg. Control
OPTICAL DENSITY
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
GROWTH TIME (HOURS)

Figure 6

of α-ketoglutaric acid and alanine were effective the addition of pyridoxyl-phosphate to this mixture further increased the growth rate. Since these observations are consistant it is probable that transaminase was not the point of the inhibition.

Exploration of the glutamic dehydrogenase reaction was less difficult. The addition of NH_4^+ or of DPNH to the growth medium did not reverse the inhibition. Partially purified glutamic dehydrogenase from beef liver was commercially available (Sigma). The effect of adding the enzyme suspension to the growth medium has been shown in Figure 8. The glutamic dehydrogenase had no effect on the unexposed control organisms but did give reversal of the inhibition for the exposed ones. These experiments were not without complication. The early increase and decrease in optical density was accompanied by the formation of an agglomerated precipitate which resulted from the addition of the enzyme to the growth medium.

The curve for the glutamic dehydrogenase marked pre-ppt. was an attempt to avoid this situation by premixing the enzyme and the growth medium, removing the precipitate and using only the supernatant fluid. However, without incubation the precipitate did not form and previous mixing of the medium and enzyme served only to increase the effect. In either event the reversal of the inhibition by the addition of glutamic dehydrogenase itself was consistant and clear cut. The apparently longer lag time for the reversal by glutamic dehydrogenase as compared to the addition of glutamic acid may represent the lag necessary for glutamic acid to form. However, these data were obtained from different experiments so such comparisons should be treated with caution.

For single exposures of E. coli no mutants have been observed. The effects of repeated exposures is being explored. This will give indication

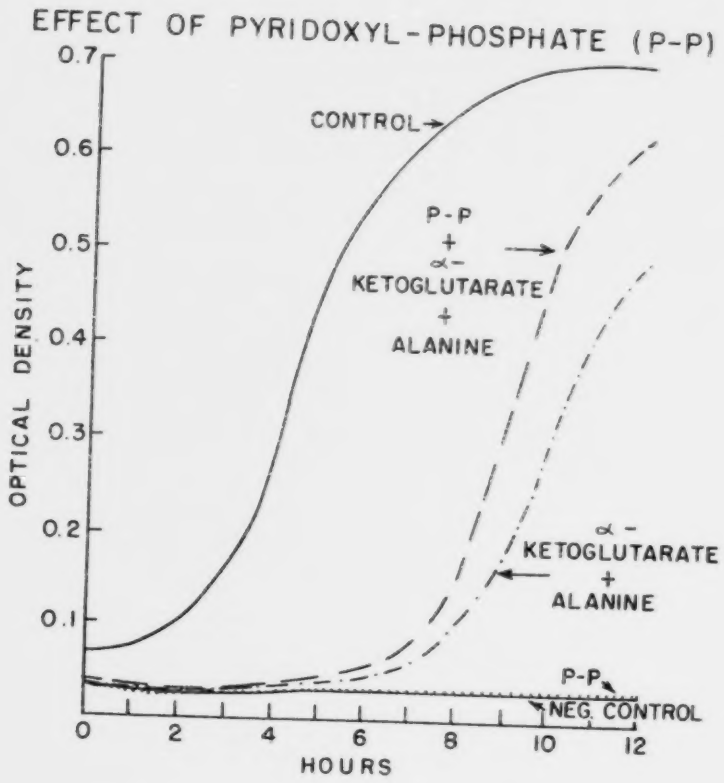

Figure 7. Effect of pyridoxyl-phosphate on the growth of E. coli following exposure to simulated oxidant type air pollutants. Exposure time 120 minutes.
Incubated in C-medium with 0.5 mmoles of alanine and α-ketoglutaric acid added as indicated. 100 γ of pyridoxyl-phosphate in water solution (filter sterilized) added as indicated.

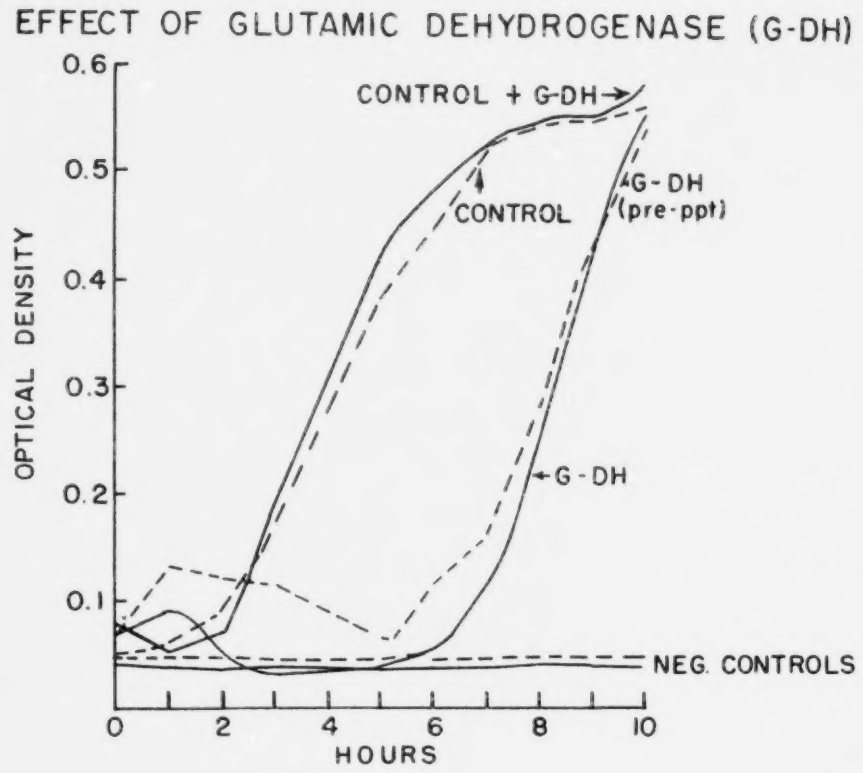

Figure 8. Effect of added glutamic acid dehydrogenase on the growth of E. coli following exposure to simulated oxidant type air pollutants. Exposure time 120 minutes.
Incubated in C-medium with 2.5 mg of glutamic acid dehydrogenase in Na_2SO_4 suspension added as indicated. Pre-ppt. glutamic dehydrogenase diluted with C-medium prior to addition but equivalent to 2.5 mg added to growth vessel.

of cummulative effects, i.e. whether the system can be permanently destroyed and if other systems are subsequently affected.

In those experiments in which the negative controls were observed for longer periods of time, recovery of growth occurred 24 to 48 hours after exposure. These differences together with previous observations that the magnitude of the inhibition of growth of E. coli bears no simple relationship to the chemical analysis of the gas mixture again suggested that there are variations in the pollutant mixture not detected by the present means of chemical analysis.

These findings are important in that a specific system has been affected; determination of the chemical groups involved, would lead to the reactant causing the effect.

The present working hypothesis involves oxidation of sulfhydral groups of the enzyme. Glutamic dehydrogenase from calf liver, has 3 SH groups per protein sub-unit (Hellerman, Schellenberg and Reiss, J. Biol. Chem. 233, 1468, 1958). If these groups are involved the observations could be from a direct oxidant effect. This is important since the magnitude of the inhibition of E. coli growth showed no correlation with oxidant analysis by the KI or phenolphthalin method. Hence this reaction may be a more discriminating method for measuring oxidants as well as biologically effective oxidant material.

C. The Effect of the Pollutant Mixture on Aldolase Activity

The demonstration of decrease in the rate of multiplication and oxygen consumption of intact E. coli following exposure to the synthetic air pollutants indicated the desirability of studying its effects on specific enzyme systems. Aldolase from E. coli was selected as the point of departure for examining the effects on enzymes of the glycolytic cycle.

The method of Sibley and Lehninger (J. Biol. Chem. 177, 859, 1949) was used to determine aldolase activity. This method involved the use of fructose diphosphate (FDP) as substrate, trapping the resultant triose phosphate with hydrazine, and determining the optical density at 540 mμ.

Examination of various conditions in the method indicated that for standardization of FDP the molarity could be determined from a standard curve of fructose concentration and that the Spectronic 20 gave as consistant and reproducible readings as did the Coleman Jr. Spectrophotometer.

The cell-free extracts of E. coli were prepared from a 24-hour broth culture of E. coli strain K-12, centrifuged at 12,800 x g and 0°. The cells were washed twice with saline, and once with sterile water. The washed cells were suspended in 25 ml of sterile water and the suspended cells ruptured in a sonic oscillator operated at full capacity for 15 minutes (Raytheon Model DF-101, 250 watts, 10 Kc.). The ruptured cells mixture was centrifuged at 25,000 x g for 25 minutes at 0°. The supernatant fluid was kept frozen. For aldolase determinations working concentrations were prepared by dilution of the cell-free extract. Relative activity has been expressed on the basis of the diluted preparation.

For exposure to the simulated pollutant mixture, 10 ml of the cell-free extract were used in a rotating cylindrical chamber of the type shown in Figure 9. The temperature within the chamber was kept at around 20° C by placing an ice bath under the exposure chamber so that half the chamber was always immersed.

The effect of exposure to the irradiated and unirradiated effluent lines on aldolase activity has been shown in Figure 10. The increased effectiveness resulting from irradiation was most evident for 15 minute exposures.

The observation that zinc, iron, cobalt and copper reverse the cysteine inhibition of aldolase from yeast (Warburg and Christian, Biochem. Z. 314, 149, 1943: Christian in Methods of Enzymology, Colowick and Kaplan, Editors, Academic Press, New York, 1955, Vol. 1, p. 317) led to the examination of the possibility that metals might reverse the inhibition of the pollutant mixture.

The effect of 20 minute exposure to the pollutant mixture on the aldolase activity of E. coli in the presence and absence of selected divalent metals is shown in Table III. From these data it would appear that zinc, and cobalt definitely enhance the activity of unexposed aldolase. These observations are summarized in Table IV, and raised the question of whether the effect of the metal ion was on the method of determination, on the enzyme, or on a component of the mixture.

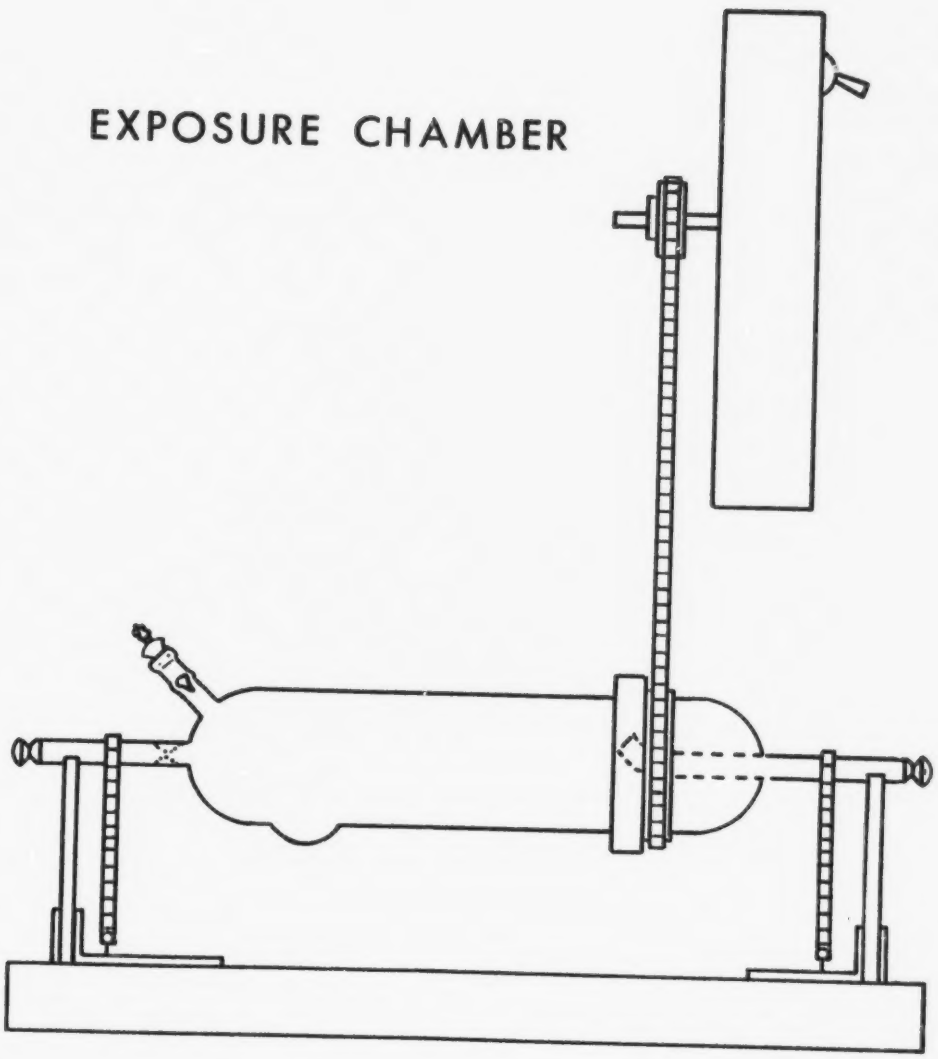

Figure 9

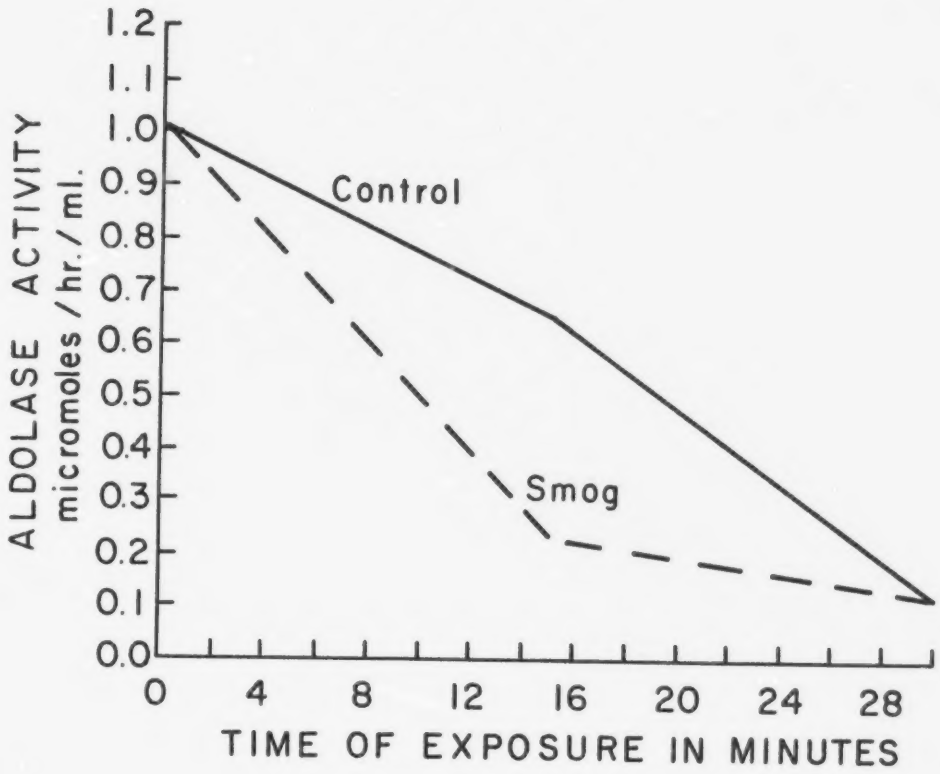

Figure 10. The effect of irradiated (smog) and unirradiated (control) lines on aldolase activity of E. coli.

TABLE III

THE EFFECT OF METALS ON THE ACTIVITY OF ALDOLASE AFTER EXPOSURE TO POLLUTANT MIXTURE

METAL* ADDED AFTER EXPOSURE		ALDOLASE ACTIVITY* micromoles/hr/ml EXPOSED CONTROL	UNEXPOSED CONTROL
Mn	0.45	0.06	0.36
	0.75	0.03	0.87
Mg	-0.08	0.06	0.06
Zn	1.29	0.20	0.30
	0.87	0.03	0.87
Cu	0.09	0.20	0.30
Ni	0.12	0.09	0.60
Co	0.87	0.03	0.87
	1.66	0.09	0.60
	0.75	0.03	0.84
Fe^{+++}	0.00	0.00	0.50
Fe^{++}	0.00	0.00	0.50

All exposures 20 minutes

* Metal ions added as aqueous solutions of sulphate in all experiments 50 millimoles metal ion added.

* Micromoles FDP split/hr/ml aldolase preparation.

TABLE IV

EFFECT OF METAL IONS ON ALDOLASE ACTIVITY

Metal ion	Aldolase Activity* micromoles/hr/ml			
none (control)	0.87	0.60	0.36	0.50
Mn	0.60		0.52	
Mg			0.24	
Zn	2.48		1.23	
Co	2.56	1.47		
Ni		0.00		
Cu			0.12	
Fe^{++}				0.17
Fe^{+++}				0.15

Metal ions added as aqueous solution of sulphate. In all experiments 50 millimoles metal ion added to 3.5 ml incubation mixture.

* Micromoles FDP split/hr/ml aldolase preparation.

D. Examination of the Effects of Metals and Chelating Agents on Aldolase Activity.*

Aldolase from muscle and from yeast have been reported to have somewhat different characteristics. Thus, for crystalline muscle aldolase, Warburg and Christian reported no inhibition by complexing agents and no requirements for coenzymes (Biochem. Z. 314, 149, 1943). However, it has been reported to be inhibited by heavy metals, notably silver and copper (Herbert et. al.,Biochem. J. 1108, 1940). Conversely yeast aldolase has been reported to be inhibited by complexing agents and this inhibition is reversed by the addition of metals. Zinc, iron, cobalt and copper reverse cysteine inhibition (Warburg and Christian, op cit and Christian in Methods of Enzymology, Vol. I, p. 317). Even in preparations of aldolase from yeast which had activities comparable to crystalline aldolase from muscle the addition of Zn^{++}, Fe^{++}, or Co^{++} was required for activity. This was explained by assuming that the yeast preparation contained an inhibitor which was inactivated by the traces of metals (O. Meyerhof, in The Enzymes, Vol. II, part I, p. 162, Academic Press, New York, 1951).

The differences in aldolase from these sources precluded assumptions about the aldolase activity of E. coli. Hence examinations of the effects of metals and complexing agents were undertaken. For comparison the effects of these materials on muscle aldolase (Boehringer and Sons) was also determined.

Confirmation of the validity of the observations required assurance that the effect of the metal ion was not on the method of determination.

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* Presented in part at the 139th Meeting of the American Chemical Society, St. Louis, March, 1961.

The method used for determining aldolase activity involved the trapping of the trioses formed as the 2,4-dinitrophenyl derivatives; methyl glyoxal-2,4 dinitrophenyl osazone and pyruvic-2,4-dinitrophenyl-hydrazone (Beck, J. Biol. Chem. 212, 847, 1955). For examination of the effects of the metal on the determination, dihydroxyacetone (DHA) was substituted for fructose diphosphate and aldolase. The difference in absorbance in the absence (reagent blank) and in the presence of DHA represents the absorbance of the hydrazone formed in the reaction. Comparison of the differences with that found in the presence of the metal should give indication of the effect of the metal on the reaction. At the concentration of metal ions used for the experiments in Table IV several of the ions showed definite absorbance at 540 mμ and appeared to have a possible effect on the hydrazone formation. Examination of the effects at various metal and DHA concentrations has been shown in Table V. At all concentrations manganese ion reduced the change in absorbance. This was a reflection of the higher absorbance in the absence of DHA (blank) which increased with manganese concentration. Those experiments with manganese also showed perceptible differences in color from the control determination. Nickel and copper also gave a higher absorbance in the blank. Thus for these ions it is not valid to assume that the inhibition indicated in Table IV was an effect on the aldolase reaction.

Since the DHA formed from the aldolase reaction must be a function of time, the lower DHA concentration shown in Table V probably better indicated the actual conditions during the determination. Comparison of these concentrations also suggested that a lower concentration of aldolase, such that the total activity was small, would further help to eliminate possible effects of the metal on the determination.

TABLE V

EFFECT OF METAL IONS ON THE ALDOLASE DETERMINATION
(no enzyme)

	millimoles added	Change in Absorbance in presence of DHA 0.6 micromoles		1.25 micromoles	
Control		0.18	0.23	0.52	0.55
Zn	.025	0.24	0.29	0.51	0.48
	.050	0.19	0.26	0.57	0.38
	.075	0.12	0.24	0.49	0.40
Co	.025	0.14	0.20	0.43	0.40
	.050	0.19	0.19	0.37	0.43
	.075	0.09	0.18	0.32	0.29
Mg	.025	0.24		0.52	
	.050	0.27		0.58	
	.075	0.25		0.54	
Mn	.025		0.03		0.18
	.050		0.01		0.05
	.075		-		0.05
Fe^{++}	.050		0.21		
Ni	.050		0.12		
Cu	.050		0.00		

Metal ions added as aqueous solution of the sulfates to 3.5 ml of incubation mixture.

A series of determinations on the same preparation over a period of 11 days, gave the data presented in Table VI. For this preparation the variation in the controls was not a function of the age of the preparation. Comparison of the activities indicated that zinc definitely enhanced the activity of aldolase from E. coli while cobalt does not.

Although cysteine had been shown to inhibit yeast aldolase (Warburg and Christian, op cit) it did not inhibit aldolase from E. coli. This has been shown in Table VII. In combination with cobalt (Table VIII) or with zinc (Table IX) the highest cysteine concentration gave some indication of stimulation of aldolase activity. Similar observations were made with magnesium and manganese. In combination with cobalt this was not true for the lower cysteine concentrations (Table VIII). These experiments indicated that cysteine definitely did not inhibit aldolase from E. coli and raised the question of the possible inhibition by other complexing agents.

The presence of ethylene-diamine-tetraacetate (EDTA) was markedly inhibitory to the aldolase activity. The data in Table X further indicated that there was little or no recovery of activity when the EDTA-metal ion ratio was 5:1. At an EDTA-metal ratio of 1:1 the presence of the metal ions gave a partial recovery of activity.

For comparison, the effect of cysteine and EDTA on the activity of aldolase from rabbit muscle was examined. For this purpose "Boehringer" analytical reagent grade preparation obtained from Sigma was used. With this preparation EDTA gave excellent duplication of the control values, indicating that it was without effect on aldolase activity. This was also true of cysteine. Table XI further indicated that the presence of EDTA enhanced the activity of the

TABLE VI

EFFECT OF ZINC AND COBALT ON THE ACTIVITY OF ALDOLASE FROM E. COLI

	Aldolase Activity, micromoles/hr/ml		
	Control	Zn^{++}	Co^{++}
	0.64	1.49	0.62
	0.84	1.88	1.04
	0.59	1.34	0.80
	0.67	1.47	0.95
	0.77	1.62	0.90
	0.95	1.52	0.59
	0.97	2.55	1.03
	0.59	1.31	0.51
	0.77	2.27	0.85
Mean	0.754	1.716	0.81
S.D.	0.144	0.433	0.195
"t"		5.53	0.5358
"t" 1% Probability		4.015	

Aldolase preparation 1 to 11 days old.

Zn^{++} and Co^{++} added as the sulfate (0.1 mM) final concentration in the incubation mixture 14 micromoles.

TABLE VII

EFFECT OF CYSTEINE ON THE ACTIVITY OF ALDOLASE FROM E. COLI

	Aldolase Activity, micromoles/hr/ml			
	Control	+ Cysteine		
		140*	70*	14 *
	0.77	1.70		
	0.97	0.77	0.85	
	0.59	0.57	0.36	0.54
	0.77	0.90	0.77	1.03
	0.92	0.92		0.90
Mean	0.80	0.97	0.66	0.81
S.D.	0.149	0.442		
"t"		0.527		
"t" 50% Probability		0.727		

*Final concentration of cysteine, micromole/ml of incubation mixture.

Aldolase activity given in micromoles/hr/ml.

TABLE VIII

EFFECT OF COBALT WITH CYSTEINE ON ALDOLASE ACTIVITY
(micromoles/hr/ml)

Control	Co^{++} 14*	Cysteine 140*	Cysteine + Co^{++} (10:1)**
0.77	0.90	1.70	1.80
0.97	1.03	0.77	1.44
0.59	0.51	0.57	0.77
0.77	0.85	0.90	0.87
		70 *	(5:1)
0.97	1.03	0.77	0.95
0.59	0.51	0.36	0.57
0.77	0.85	0.77	0.67
		14 *	(1:1)
0.59	0.51	0.54	0.67
0.77	0.85	1.03	0.69

* Concentration in the incubation mixture, micromoles/ml.

** Mole ratio of cysteine to cobalt in incubation mixture.

TABLE IX

EFFECT OF ZINC WITH CYSTEINE ON ALDOLASE ACTIVITY
(micromoles/hr/ml)

Control	Zn 14*	Cysteine 140*	Cysteine Zn (10:1) **
0.77	1.62	1.70	3.04
0.97	2.55	0.77	2.83
		70*	(5:1)
0.59	1.31	0.36	1.50
0.77	2.27	0.90	1.20
		14*	(1:1)
0.65	1.49	0.49	1.67
0.84	1.88	0.62	1.78

* Concentration in the incubation mixture, micromole/ml.

** Mole ratio of cysteine to zinc in the incubation mixture.

TABLE X

EFFECT OF EDTA ON ALDOLASE ACTIVITY OF E. COLI

Metal	Aldolase Activity, micromoles/hr/ml				% Inhibition (14 micromoles/ml)
	Control	EDTA 70*	Control	EDTA 14*	
none	0.67	0	0.95	0.05	99
Zn^{++}	1.47	0.15	1.52	1.11	27
Co^{++}	0.95	0.05	0.59	0.36	39
Mg^{++}	0.63	0.08	0.69	0.10	85
Mn^{++}	0.33	0	0.31	0.18	42

Metal ion added as the sulfate salt. Final concentration in incubation mixture 14 micromoles/ml.

* Micromoles of EDTA/ml of incubation mixture.

TABLE XI

EFFECT OF EDTA WITH METALS ON ALDOLASE ACTIVITY
FROM RABBIT MUSCLE

	Aldolase Activity, micromoles/hr/ml				
	No metal	Zn	Co	Mg	Mn
Control	1.09	0.95	0.28	1.03	0.75
EDTA (140)	1.15	1.03	0.85	1.05	0.98
Control	0.67	0.69	0.15	0.72	0.26
EDTA (70)	0.67	0.75	0.51	0.80	0.59
Control	0.93	0.82	0.23	0.75	0.28
EDTA (14)	0.93	0.90	0.49	0.95	0.28

EDTA concentrations shown are that of incubation system, micromoles/ml.

Metals as sulfates, concentration in incubation mixture 14 micromoles/ml.

aldolase in the presence of cobalt. This enhancement was apparent at an EDTA-cobalt ratio of 1:1 suggesting that cobalt may be an inhibitor for aldolase from rabbit muscle.

A series of experiments examining the effects of the metal ion on the activity of aldolase from rabbit muscle was summarized in Table XII. Magnesium had no effect, and the apparent effect of zinc was not statistically significant. The inhibition of cobalt was statistically significant. The apparent inhibition of manganese was not regarded as real since at this concentration manganese had an inhibitory effect on the determination (Table V). This was also reflected in the absence of an effect at an EDTA-manganese ratio of 1:1 (Table XI).

The effect of cobalt on the activity of aldolase from rabbit muscle was further examined at several cobalt concentrations as shown in Table XIII. Cobalt was inhibiting and the magnitude of the inhibition appeared to be a function of concentration of the cobalt. However at similar aldolase activity of the control the magnitudes of the inhibition at a particular cobalt concentration are quite different. This has been shown in Figure 11.

Similar studies with the addition of zinc at various concentrations has been shown in Table XIV. Except for one experiment zinc lowers aldolase activity but the effect is not a function of zinc concentration. It should be noted that neither of the aldolase preparations used for these experiments were represented in Table XII.

The inhibition of the muscle aldolase by cobalt leads to examination of the combined effects of cobalt and cysteine shown in Table XV. At the higher aldolase activity the higher concentration of cysteine appeared to be stimulating. This is not true at the lower

TABLE XII

EFFECT OF METALS ON ALDOLASE FROM RABBIT MUSCLE

	Aldolase Activity, micromoles/hr/ml				
	Control	Zn	Co	Mg	Mn
	0.93	0.82	0.23	0.75	0.28
	1.15	0.95	0.28	1.03	0.75
	0.67	0.49	0.23	0.90	0.05
	0.44	0.15	0.10	0.39	0.28
	0.67	0.69	0.15	0.72	0.26
Mean	0.77	0.62	0.20	0.76	0.32
"t"			4.704		
"t" 1% probability			4.604		

TABLE XIII

EFFECT OF COBALT ON THE ACTIVITY OF ALDOLASE FROM RABBIT MUSCLE

Aldolase Activity, micromoles/ml/hr.						
Control	Cobalt*					
	75 µmoles	50 µmoles	25 µmoles	20 µmoles	15 µmoles	10 µmoles
1.55		0.23		0.44		0.75
3.04	0.41	0.57	0.85	3.04		
			0.08	0.39	1.06	0.57
3.22	1.93	2.52	3.09			
0.93		0.23				
1.09		0.28				
0.69		0.23				
0.44		0.10				
0.67		0.15				
3.30	1.42	1.47				

Cobalt added as 0.1 mM solution of sulfate.

* Micromoles present in 3.5 ml of incubation mixture.

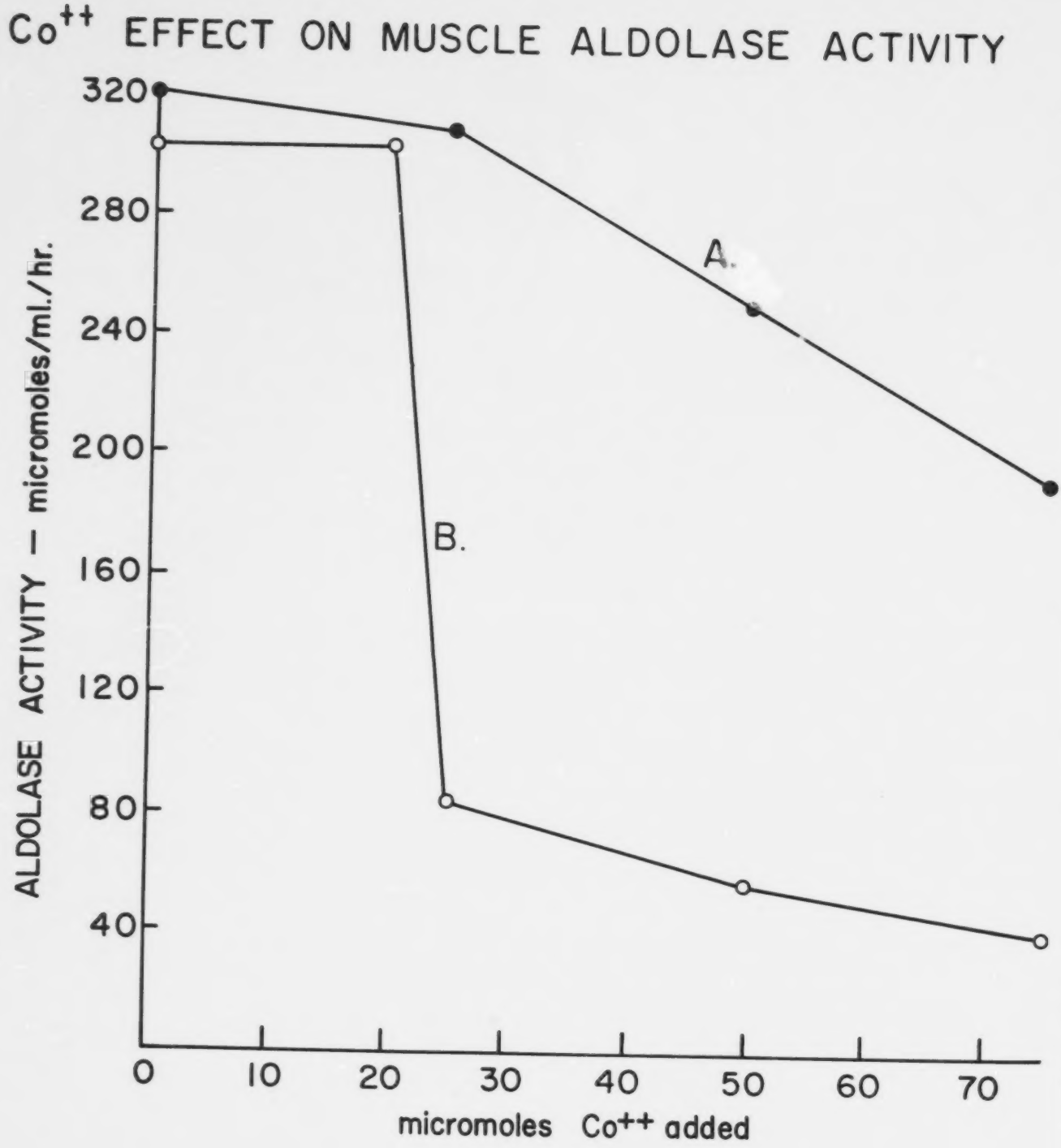
Co++ EFFECT ON MUSCLE ALDOLASE ACTIVITY
ALDOLASE ACTIVITY — micromoles/ml./hr.
320
280
240
200
160
120
80
40
A.
B.
0
10
20
30
40
50
60
70
micromoles Co++ added

Figure 11

TABLE XIV

EFFECT OF ZINC ON THE ACTIVITY OF ALDOLASE FROM RABBIT MUSCLE

Aldolase Activity, micromoles/ml/hr						
Control	Zinc*					
	75 µmoles	50 µmoles	25 µmoles	20 µmoles	15 µmoles	10 µmoles
1.55	0.62	1.24	1.06	0.59	1.03	0.70
0.62	0.23		0.44			0.72
1.08	0.75	0.64	1.18	0.85	1.21	
2.01	2.47	2.29	3.86			
0.93		0.82				
1.09		0.95				
0.69		0.49				
0.44		0.15				
0.67		0.69				
3.30	2.96	2.91	2.88			

Zinc added as sulfate (0.1 mMolar solution)

* Micromoles present in 3.5 ml of incubation mixture.

TABLE XV

EFFECT OF COBALT AND CYSTEINE ON THE ACTIVITY OF ALDOLASE FROM RABBIT MUSCLE

Aldolase Activity, micromoles/ml/hr			
Control	Cobalt		
	No Co	75 mμmoles	50 mμmoles
	500 μmoles cysteine:		
3.22	4.07	4.04	4.02
0.44	0.36		0.46
	50 μmoles cysteine:		
0.69	0.64		0.44
3.22		1.98	

Cobalt added as sulfate (0.1 mMolar solution)

Cysteine (0.1 mMolar solution) 500 or 50 umoles added to 3.5 ml incubation mixture.

activity or lower cysteine concentration. From these few experiments it appeared that at the lower cysteine concentration was not able to overcome the presence of even 50 μmoles of cobalt.

The effect of the combination of zinc and cysteine shown in Table XVI, indicated that cysteine did not overcome the erratic effect of the zinc seen in Table XIV.

The studies of the effects of the divalent metals on aldolase activity indicate that the activity of aldolase from E. coli is enhanced by the presence of zinc and probably cobalt, inhibited by EDTA but not by cysteine. Conversely aldolase from muscle was inhibited by cobalt and possibly by zinc, but not by EDTA or cysteine.

The contrast in the effect of the metals on aldolase activity from these sources may represent differences in extraneous materials present in the preparations. However, such differences have been previously noted with respect to yeast and muscle preparations. Thus the data lends further support to the possibility that there exists different arrangement of groups around the enzymatic site for the aldolase from the two sources.

TABLE XVI

EFFECT OF ZINC AND CYSTEINE ON THE ACTIVITY OF ALDOLASE FROM RABBIT MUSCLE

Aldolase Activity, micromoles/hr/ml					
Control	Zinc				
	No Zn	75 µmoles	50 µmoles	25 µmoles	10 µmoles
	cysteine* 140 micromoles/ml				
0.62		0.36			0.59
2.01	2.78	4.09	3.86	2.27	
0.44	0.36		0.62		
3.32	3.68	3.48	3.45	4.20	
	cysteine* 14 micromoles/ml				
0.69	0.64		0.41		
0.62		0.21		0.23	0.15
2.01		1.21	3.86		
3.22			1.98		

Zinc added as sulfate (0.1 micromoles solution)

Cysteine (0.1 micromoles solution) 500 or 50 µmoles added.

* Final concentration in the incubation mixture.

E. The Effect of pH on Aldolase Activity

In general, exposure of the aldolase suspension to the pollutant mixture lowered the pH of the suspension.

Preliminary experiments had indicated little difference in aldolase activity for suspensions at pH 4.0 as compared to pH 7.0. However increasing the concentration of the tris buffer in which the cell free extract was suspended, reduced the magnitude of the inhibition by the pollutant mixture. This is shown in Table XVII. From experiments of this type it is evident that at the lower pH the aldolase activity is reduced. However, decreasing the pH alone, as with HCl, is somewhat less effective than exposure to the pollutant mixture and even with the higher concentration of the buffer, 45% of the activity has been lost after a 20 minute exposure. These observations indicate that more than acidity is involved in the loss of aldolase activity.

In Table XVIII is a comparison of the effect of acidity in the presence of zinc. The enhancement of aldolase activity for the unexposed cell free extract at pH 7.5 is evident as is the decreased activity at pH 4.0. For the exposed aldolase the addition of zinc restores a major part of the activity which was further increased by readjustment of the pH to 7.0. The combination, however, did not increase the activity to that of the unexposed aldolase in the presence of zinc. Experiments of this type indicate that the effect of the pollutant mixture is at least two fold.

TABLE XVII

COMPARISON OF BUFFER CONCENTRATION FOR EXPOSURE OF ALDOLASE TO THE POLLUTANT MIXTURE

	pH after treatment	Aldolase activity micromoles/ml/hr
0.2 M Buffer:		
Exposed	7.5	0.71
Unexposed	7.0	0.39
0.1 M Buffer:		
Exposed	3.0	0.71
Unexposed	7.5	0.00
Unexposed + HCl	3.0	0.17

Buffer: Tris (hydroxymethyl) aminomethane

Exposure to pollutant mixture - 20 minutes

Analysis of Effluent:

Oxidant (μg O_3/l) - 6.8
NO_2 (ppm NO_2/l) - 0.32
Acid (meq/l) - 0.029
302 mμ absorbing material (mg NO_3/l) - 2.23

TABLE XVIII

COMPARISON OF ACID AND ZINC ION ON ALDOLASE ACTIVITY
AFTER EXPOSURE TO THE POLLUTANT MIXTURE

	EXPOSURE TIME (minutes)	pH	ALDOLASE ACTIVITY micromoles/ml/hr
no metal	0	7.5	1.10
Zn	0	7.5	2.90
Zn + HCl	0	4.0	0.12
no metal	20	4.5	0.34
Zn	20	4.5	1.57
Zn + NaOH	20	7.0	2.02

Zinc added as aqueous sulfate (50 mumoles added)

Analysis of Effluent:

Oxidant ($\mu gO_3/l$) - 6.2
NO_2 (ppm NO_2/l) - 0.29
Acid (meq/l) - 0.21
302 mμ absorbing material (mg NO_3/l) - 2.28

F. Gas Chromatographic Studies.

Except for limited preliminary studies, comparison of the chemical analysis and of biological activity with the gas chromatographic analysis was precluded by the necessity for completely replacing the wiring in the 1954 "Kromo-tog". The original design of this instrument enclosed all electrical circuits in a single unit. This compactness made repairs difficult and, as the insulation aged, less and less shielding of one circuit from another was provided. Although there had been progressively more "downtime" the basic problem did not reveal itself until the induced current became sufficient to produce a signal when the detector cell was removed. The choice became rebuilding the instrument or completely scrapping it.

By rebuilding the instrument using modular assembly, it was possible to incorporate previously constructed inovations such as the gas carrier control manifold, into the control panel, and to have the controls for each column completely separated. The wiring diagrams for each of the panels have been included in the appendix. With these modifications this instrument can serve for line monitoring. Because of the time required to obtain the components these repairs have taken considerably longer than was anticipated. Hence the instrument has not been returned to operation.

Previous investigation had shown that a Carbowax 1500 (Union Carbide) column which has a relative long retention time for water, gave a crude separation of the pollutant mixture of at least eight peaks more rapidly eluted than water.

Examination of the retention volumes of various substances led to the observation that mineral acids could not be quantitatively eluted

from the columns and that the retention properties of the column appeared to change gradually when acid containing samples were analyzed. This observation led to exploring for a column packing material which would effectively remove acids from the gas samples without removing other polar compounds which might be present. Studies on C-22 Firebrick, 30-60 mesh, coated with 20% (w/w) disodium phosphate packed in 5 cm. tubing (7 mm I.D.) indicated quantitative removal from an air stream of organic as well as inorganic acids as represented by: hydrochloric, phosphoric, nitric, acetic, propionic and butyric acids. Of the other systems similarly prepared for acid removal, the order of efficiency was sodium hydroxide < sodium bicarbonate < sodium carbonate < trisodium phosphate.

The disodium phosphate-Firebrick column also permitted only semi-quantitative recovery of representative alcohols, ketones and nitro-hydrocarbons having boiling points of 132° or less. Thus these compounds are in part held by the column.

The use of the disodium phosphate-Firebrick column in the effluent line before exposure of E. coli preparations to the pollutant mixture eliminated the inhibition on growth of E. coli and on aldolase activity. (For details of these experiments see the following section of the report.) This suggested that the biologically active material present in the pollutant mixture was either adsorbed on, or destroyed by the column as a result of the large surface area presented to promote surface reaction.

Attempts to elute possibly adsorbed materials from the disodium phosphate-Firebrick column by raising the temperature of the column and flushing with carrier gas proved unsuccessful. It is possible that the temperature required to remove the adsorbed materials are sufficient to

produce reaction and degradation. Unfortunately, equipment limitations do not permit a conclusive answer to this question. The design of the gas handling system on the "Kromo-tog" is such that gas samples entering the instrument must pass through several feet of unheated piping before reaching the analytical column. This piping cannot be thermally regulated at sufficiently high temperatures to exclude the possibility that materials are being eluted from the trapping columns but are condensing in the gas handling system before reaching the detection system. Until such equipment is available for application to this study column trapping methods offer little promise for the solution of the problem.

If it is assumed that the active materials are retained on the column, observations on the retention of pure materials by the disodium phosphate-Firebrick column again led to the conclusion that the compounds responsible for biological activity must be of a highly polar nature.

From our previous work it has become apparent that detection of organic acids, both mono- and dicarboxylic, was essential to the analysis of the pollutant mixture as well as products of the biochemical reactions. The recent acquisition of Barbier-Colman gas chromatographic equipment (models 10 and 20) with both packed and capillary columns and with both hydrogen flame and ionization detectors permitted work on this problem (section J), as well as further evaluation of the pollutant mixture (appendix A).

G. Attempts to Remove Components of the Pollutant Mixture (A Comparison of Chemical Analysis and Biological Activity).

Previous work on analysis of a grab sample of the pollutant mixture by gas chromatography on a Carbowax 6000 column indicated that there were a minimum of 8 components in the mixture which were more rapidly eluted from the column than water. Likewise, drying the pollutant mixture with ascarite or magnesium perchlorate resulted in complete loss of biological activity of the effluent.

In exploring the inhibition of oxygen consumption of polymorphonuclear leucocytes by the pollutant mixture, a technique for indirect exposure of the cells was developed. This involved bubbling the pollutant mixture through a Ringers-phosphate solution, suspending the cells in the exposed solution for a brief period of time, and resuspending the cells in fresh, un-exposed medium for the determination of oxygen consumption. Although the inhibition was not as great as that observed by direct exposure, by this technique it was demonstrated that for these cells the inhibitor effect of the pollutant mixture was not caused by change in pH or ionic strength of the suspending medium. These observations further indicated that either a portion of the active material was water soluble or that the aerosols retained their integrity and part of their activity even in aqueous suspensions (Estes and Gast, Am. Chem. Soc. New York, September 1957).

Because of the nature of the cells it was not possible to apply this technique directly to the examination of the effects of the pollutant mixture on the growth of E. coli. Hence aliquots of the cells were exposed to the pollutant mixture directly, and after a bubbler containing 10 ml of 0.25 M phosphate buffer. For another aliquot of cells, 1 ml of

the exposed buffer was substituted for fresh buffer in preparing the medium for subsequent growth. The results have been presented in Figure 12.

For these studies it was unfortunate that growth of the negative control (exposed without bubbler) showed so little inhibition. A comparison of the analysis of the effluent for this and the following experiment, Figure 13, indicated that this was not the result of lower concentration of any component for which analysis was made.

With the bubbler in the line there was little or no effect on the growth of the organisms. Likewise, 1 ml of the exposed buffer added to 7 ml of the growth medium had no effect on the growth of the cells.

In other experiments analysis of the effluent before and after the bubbler has been summarized in Table XIX. A portion of all components had been removed. The buffer appeared to be most efficient for removing the 302 mμ absorbing material.

To determine the concentration of these materials in the absorbing solutions, methods were modified for use with these solutions. Comparison of the analysis of the gas and the solution has been given in Table XX. Most disturbing was the observation that the absorbing solution indicated higher oxidant concentration that did direct analysis of the gas. Does this mean that potential oxidant material in the gas becomes actual oxidant material after absorption into the collecting solution?

Conversely, attempts to cold trap components of the effluent gas in a trap containing glass wool to increase the surface area indicated that glass wool destroyed rather than trapped the oxidant material. In Table XXI has been shown a comparison of attempts to cold trap the oxidants in a Sheppard trap enclosed in a dry-ice-ethanol bath with and

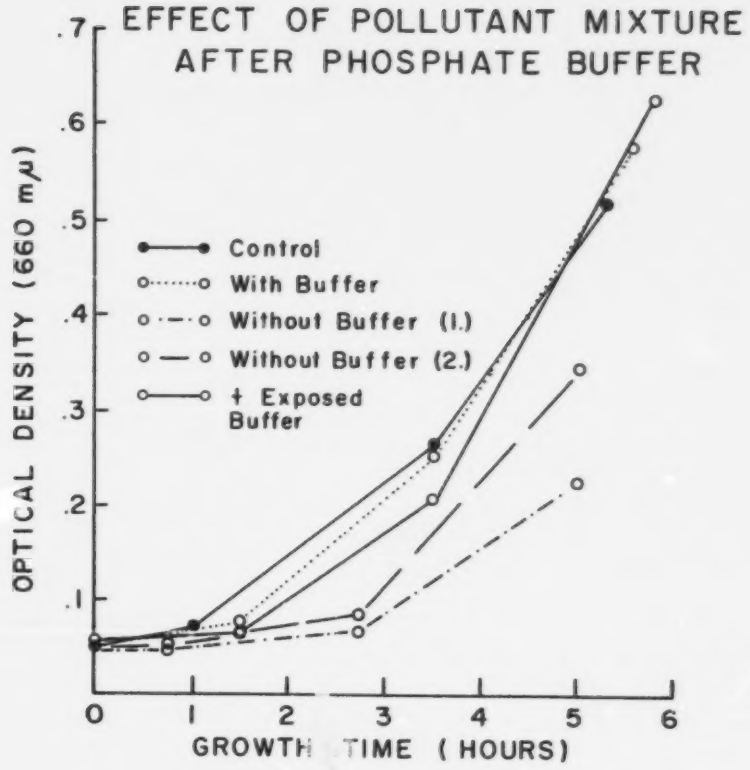

Figure 12. The Effect of the Pollutant Mixture, after bubbling through phosphate buffer, on growth of E. coli. Exposure time 60 minutes.

Analysis of the line:
Oxidant, μg O_3/l - 6.6
NO_2, ppm/l - 0.23
Acid, milliequ./l - 0.025
302 mμ as mg NO_3/l - 3.06

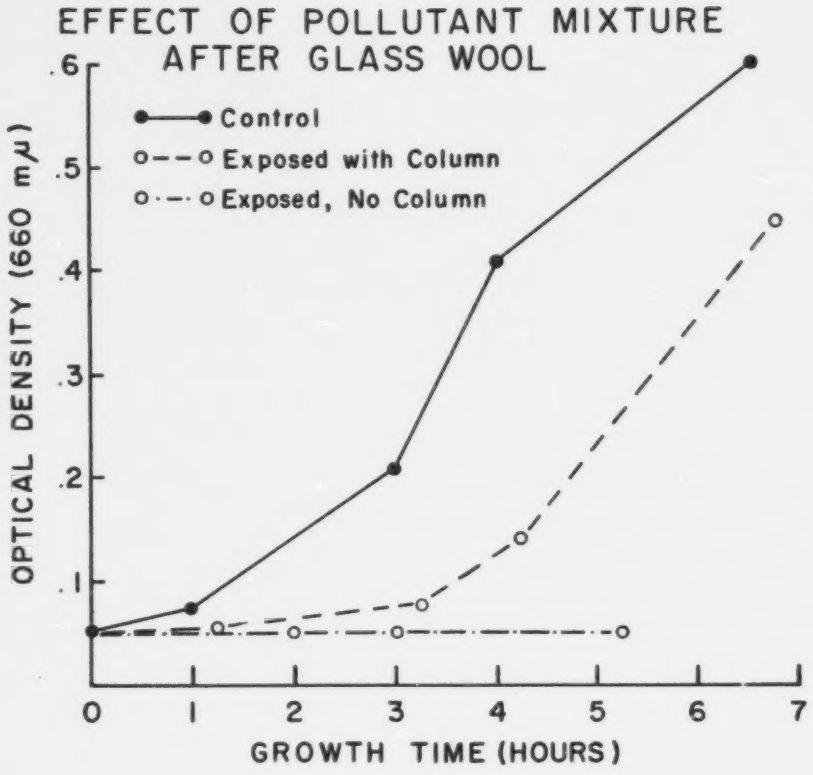

Figure 13. Effect of Glass Wool. Exposure 60 minutes.

Analysis of line:

Oxidant, μg/l - 5.8
NO_2, ppm/l - 0.36
Acid, milliequ./l - 0.030
302 mμ as mg NO_3/l - 2.79

Table XIX

EFFECT OF BUBBLING THE POLLUTANT MIXTURE THROUGH PHOSPHATE BUFFER

	Oxidant µg/1 of gas	NO_2 ppm	Acid mileq/1	302 mµ as mg NO_3/1
Direct	6.3	0.33	0.028	2.35
After Phosphate Buffer	4.6	0.12	0.002	0.00

Table XX

CONCENTRATION OF POLLUTANT MATERIALS IN GAS AND ABSORBING SOLUTION

Analysis of the gas				Analysis of the solution calculated to gas	
O_3 µg/1	NO_2 ppm	Acid mileq/1	302 mµ as mg NO_3/1	O_3 µg/1 gas	302 mµ as mg NO_3/1 gas
4.9	0.33	0.028	2.83	6.1	1.75
5.4	0.51	0.030	2.57	7.5	1.99
5.3	0.36	0.028	2.52	6.3	1.75
5.2	0.36	0.036	2.89	9.1	2.46
4.7	0.38	0.028	2.78	10.5	2.69

Table XXI

TRAPPING OXIDANT*

Glass Wool	Oxidant Trapped	Oxidant passed through trap at 5 min. intervals, interval #			
		1	2	3	Total
Absent	2.4	3.9	5.0	5.0	13.9
Present	2.2	1.9	4.4	4.5	10.8

* Reported as ug O_3/3 liters of pollutant mixture, 15 minutes collection at 200 cc/min.

without glass wool in the trap. During 15 minute (3 liters) flow of the synthetic pollutant mixture, 3 oxidant tests were made on the effluent beyond the trap. For each interval more oxidant was detected in the absence than in the presence of the glass wool, yet there was no more oxidant material detected in the trap.

For testing this effect on the growth of E. coli, a 5 cm. column of glass wool contained in 7 mm I.D. pyrex tubing was inserted into the effluent line. In these experiments the negative controls exposed directly to the pollutant mixture failed to grow while those organisms exposed behind the glass wool had demonstrated considerable recovery of growth rate.

Better recovery was observed for organisms exposed after a column of 20% (w/w) disodium phosphate on firebrick as has been shown in Figure 14. (For characteristics of this packing see Section F.)

From the analysis of the effluent before and after the column it would appear that oxidant was the most important component removed. Since these columns are not too discriminating with respect to polar materials, including nitrated and halogenated hydrocarbons, there is little indication of particular oxidant components.

Examination of the effectiveness of a disodium phosphate-Firebrick column for the protection of aldolase activity has been shown in Table XXII. Here it is evident that the column absorbed acid materials. Were this the only characteristic of these columns it might be assumed that the more effective inhibitor of aldolase activity was a decrease of pH. However, in view of previous observations regarding pH on aldolase and oxidant removed by the column a 2-fold effect is evident. In addition the magnitude of the increased activity for aldolase exposed behind the

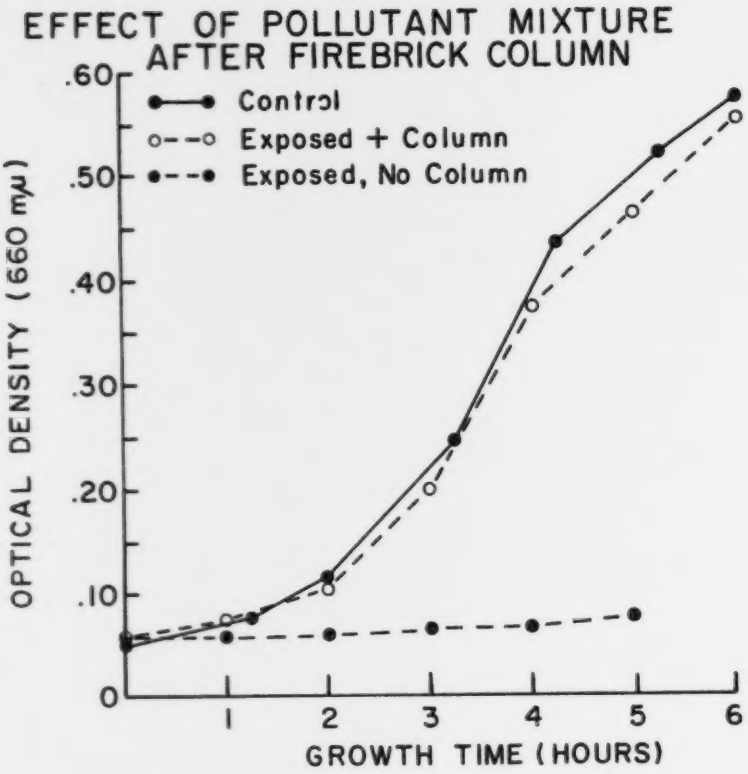

Figure 14. The Protective Effect of a Disodium phosphate-Firebrick Column on the Growth of E. coli.

Analysis of the line:

	Column present	Column absent
Oxidant, ug O_3/l	6.6	1.0
NO_2, ppm	0.31	0.59
Acid, mileq/l	0.029	0.001

Column: 5 cm. of 7 mm I.D. tubing containing C-22 firebrick, 30-60 mesh coated with 20% (w/w) Na_2HPO_4.

TABLE XXII

THE REMOVAL OF ALDOLASE INHIBITION BY A DISODIUM PHOSPHATE-FIREBRICK COLUMN

	pH	ALDOLASE ACTIVITY micromoles/ml/hr
Unexposed	7.5	0.63
Unexposed, acidified	4.0	0.00
Exposed without column	4.0	0.15
Adjusted	7.5	0.36
Exposed with column	7.5	0.92

Column: 5 cm. column of 7 mm ID tubing containing C-22 Firebrick, 30-60 mesh, coated with 20% (w/w) Na_2HPO_4.

Analysis of line: Oxidant (μg O_3/l) - 6.1
NO_2 (ppm NO_2/l) - 0.38
Acid (meq/l) - 0.027
302 mμ absorbing material (mg NO_3/l) - 2.12

column cannot be ignored and suggests that something more was occurring.

Further comparison of the analysis of the pollutant mixture after these columns has been shown in Table XXIII. These experiments represent different columns of the same dimensions and packing. Of the components tested the NO_2 appeared to be the most variable. As was previously indicated attempts to elute possibly absorbed materials from the column were unsuccessful. It is possible that with a gas chromatographic instrument in which the temperature of the sampling system can be controlled that detection of an eluate would be achieved.

Table XXIII

ANALYSIS OF POLLUTANT MIXTURE AFTER A DISODIUM PHOSPHATE COLUMN

	Oxidant ug O_3/l	NO_2 ppm	Acid mileq/l	302 mu as mg NO_3/l
Without column	6.9	0.28	0.029	2.27
With column	0.2	0.13	0.004	0.13
Without column	6.5	0.26	0.028	1.91
With column	1.2	0.25	0.007	0.89

H. Aliphatic nitro compounds and their in vitro Biochemical Effects (in conjunction with RG-9695, formerly RG-6355)

Aliphatic nitro compounds have been implicated in oxidant type air pollution, and are potential contributors to the 302 mμ absorption shown by our synthetic oxidant type pollutant mixture collected in a basic solution. Although their use as intermediates and as solvents and certain of their chemical reactions have been studied, little is known of the biochemical effects of these compounds.

Initial observations indicated that at low concentrations the aliphatic nitro compounds inhibited the oxygen consumption of leucocytes as well as homogenates of heart and liver. As the concentration of the nitro compound was increased the inhibition passed through a maximum. (Estes and Gast, Archives of Ind. Health, 1, 47, 1960) Comparison of these observations with those for exposures of leucocytes to the pollutant mixture as well as consideration of the chemistry of these nitro compounds suggested that further studies should be made and that the hydrogen transport system should be examined first.

The effects of the alkyl nitro compounds on the succinoxidase and cytochrome oxidase systems in heart tissue homogenates was examined by the method of Slater (Biochem. J. 45, 1, 1949; 82, 185, 1962). The cytochrome oxidase system was examined at three different homogenate concentrations and a single concentration of the nitro compounds. The effect of 2-nitropropane is shown in Figure 15. The variation cannot be attributed to a reaction between 2-nitropropane and either cytochrome c or ascorbic acid or a combination of these additives, since blanks of such mixtures in the absence of tissue gave no oxygen consumption and spectroscopic examination indicated no change in the absorption spectrum of cytochrome c by the addition of nitro compounds. Figure 16 shows the

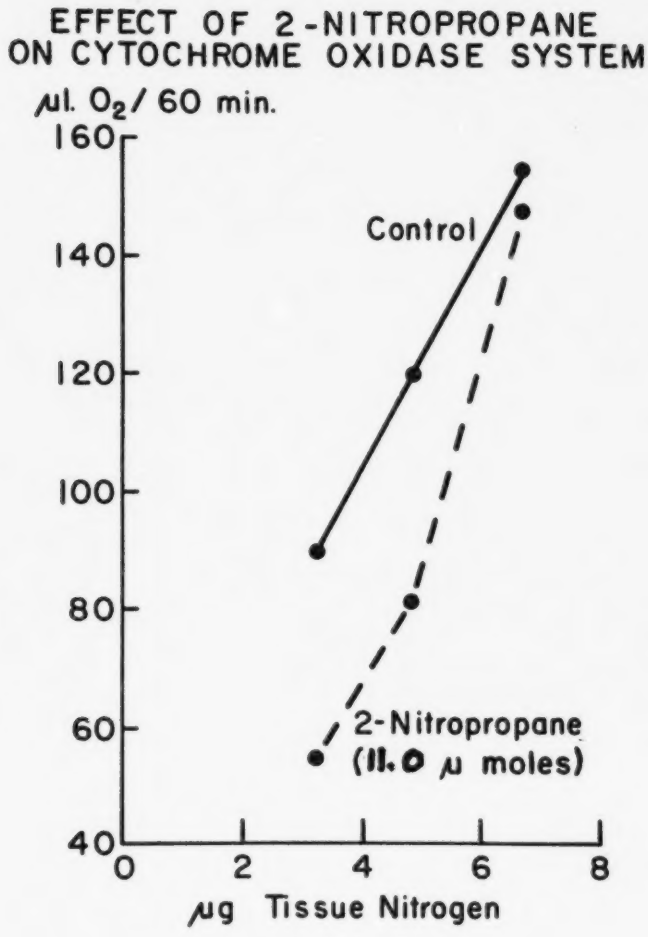

Figure 15. The Effect of 2-nitropropane on Cytochrome Oxidase System.

Cytochrome Oxidase System: 0.15 mM of phosphate buffer, pH 7.4, 0.15 M
0.20 µM of cytochrome c, 2×10^{-4} M
1.2 µM of $AlCl_3$, 4×10^{-3} M
14.2 µM of ascorbic acid, 0.114 M

2-nitropropane in phosphate buffer, pH 7.4, 11.0 µmoles/ml; tissue nitrogen equal to 0.033 mg/ml, 0.1, 0.15, 0.2 ml used. 25° C with air as the gas phase.

effect of nitroethane which was used at a similar concentration. Here the magnitude of the inhibition is relatively constant for the three tissue concentrations.

Previous observations showed that the inhibition of oxygen consumption by nitro compounds varied with the amount of tissue present, passing through a minimum, hence the succinoxidase system was examined at two tissue homogenate concentrations and three concentrations of the nitro compounds.

The effect of 2-nitropropane on the succinoxidase system is illustrated in Figure 17. The higher concentration of 2-nitropropane inhibited oxygen consumption at the higher concentration of heart tissue homogenate and stimulated it at the lower tissue concentration. For the lower concentration of 2-nitropropane the converse effect was observed. At any particular concentration of 2-nitropropane the occurance of stimulation or inhibition of oxygen consumption was in part dependant on tissue concentration. The specific activity of the particular homogenate used also appeared to be a factor. Thus with heart tissue of somewhat lower succinoxidase activity but at higher tissue homogenate concentrations, stimulation of oxygen consumption was observed for both 110.0 and 11.0 umoles of 2-nitropropane (Figure 18). As the concentration of the homogenate was increased in the presence of either concentration of 2-nitropropane, the activity did not proportionately increase. These observations suggested that two reactions were involved and that one of them could be non-enzymatic oxidation of 2-nitropropane.

The utilization of inorganic phosphate by a heart tissue homogenate in the presence of a succinoxidase system and a glucose-hexokinase acceptor system, is shown in Table XXIV. Except in the case in which the amount of 2-nitropropane relative to the tissue was at its maximum, the presence of

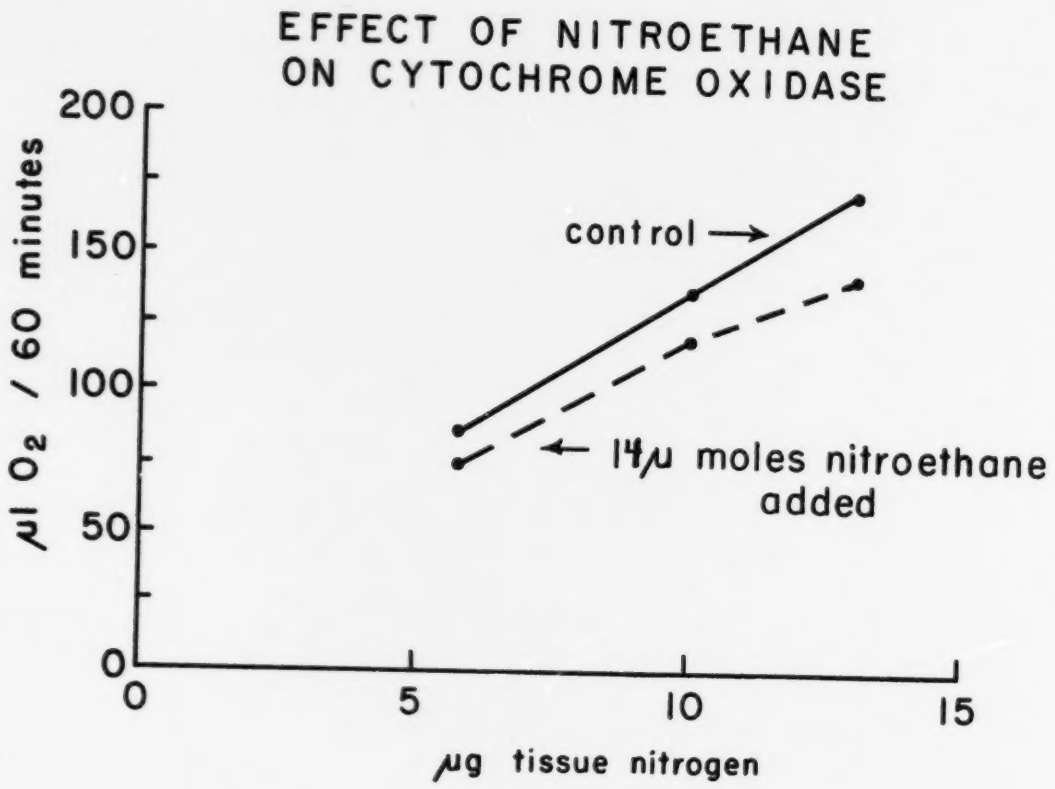

Figure 16. The Effect of Nitroethane on Cytochrome Oxidase.

Cytochrome Oxidase System:
0.15 mM of phosphate buffer, pH 7.4, 0.15 M
0.20 µM of cytochrome c, 2×10^{-4} M
1.2 µM of $AlCl_3$, 4×10^{-3} M
14.2 µM of ascorbic acid, 0.114 M

Nitroethane in phosphate buffer, pH 7.4, 14.0 umoles added. Tissue nitrogen equal to 0.066 mg/ml, 0.1, 0.15, 0.2 ml added. 25° C with air as the gas phase.

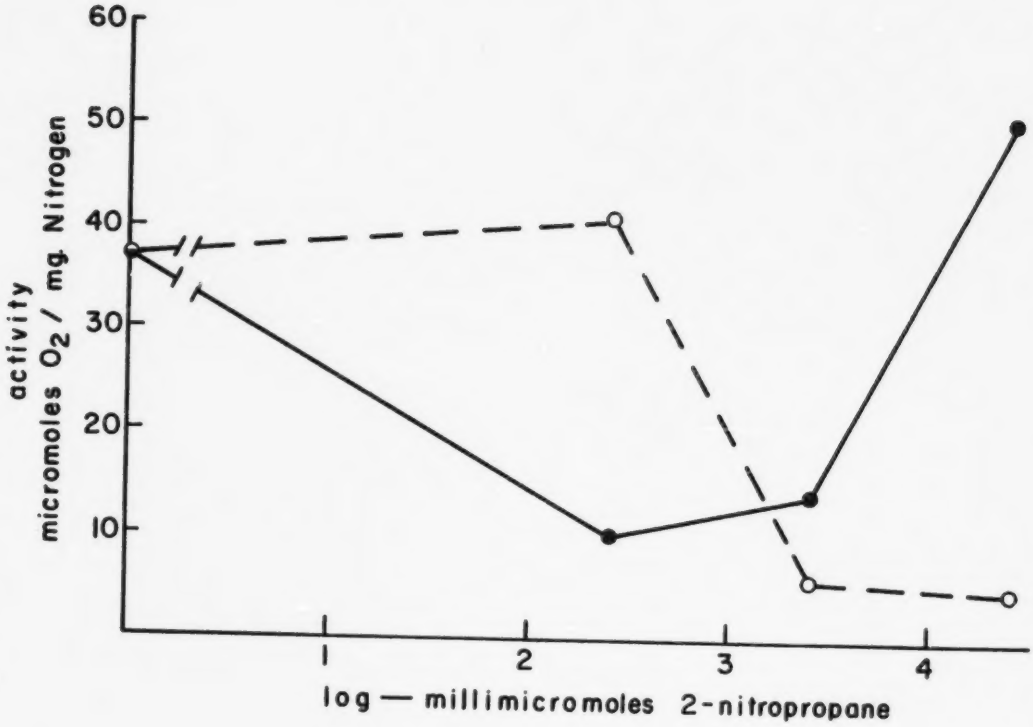

Figure 17. The Effect of 2-Nitropropane on the Succinoxidase System.

_ _ _ _ _ for 0.66 mg N. _____ for 0.132 mg N.

System: To a succinoxidase system containing:
phosphate buffer, 0.15 millimoles at pH 7.4
sodium succinate, 0.20 millimoles
cytochrome c, 0.04 micromoles
$CaCl_2$ and $AlCl_3$, 1.2 micromoles of each,
the indicated amounts of 2-nitropropane were added in phosphate buffer. For each concentration of 2-nitropropane heart tissue homogenate containing 660 and 132 µg tissue nitrogen were added. Total volume 2.8 ml. Air as the gas phase at 30° C.

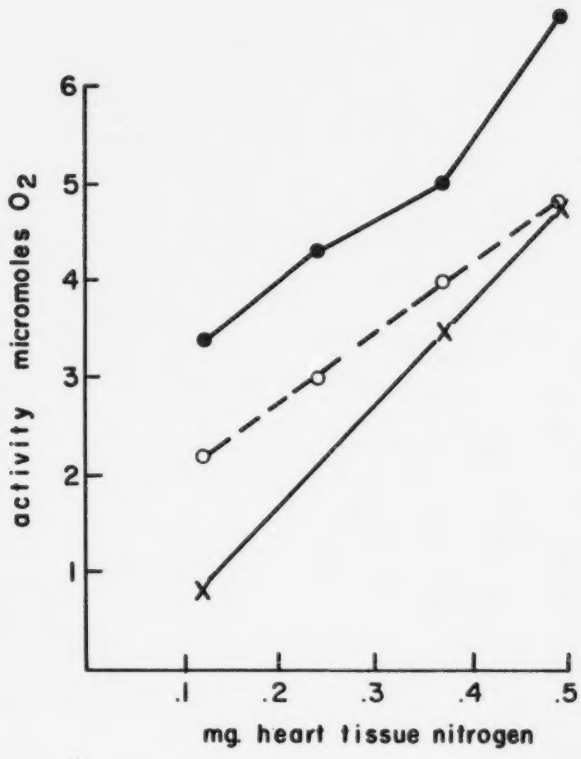

Figure 18. The Effect of 2-nitropropane on the Activity of a Succinoxidase System.

x_____x_____x no 2-nitropropane

o----------o----------o 11.0 µmoles of 2-nitropropane added

._____._____. 110.0 µmoles of 2-nitropropane added

TABLE XXIV.

DISAPPEARANCE OF PHOSPHOROUS IN THE PRESENCE OF 2-NITROPROPANE

2-nitropropane added (μmoles)	μmoles of 2-nitropropane per mg tissue N	μmoles P_i per mg tissue N	μmoles O_2 per mg tissue N	P/O
0.0	0.00	47.8	28.5	1.7
1.1	9.85	89.4	26.7	3.3
1.1	4.90	60.3	5.8	10.4
11.0	98.50	70.5	8.8	8.0
11.0	49.00	70.1	3.6	19.4
110.0	985.00	15.2	33.5	0.45
110.0	490.00	67.7	31.1	2.2

System:

Phosphate buffer, 0.15 millimoles, pH 7.4
Sodium succinate, 0.20 millimoles
Cytochrome c, 0.04 micromoles
$CaCl_2$, 1.2 micromoles
$AlCl_3$, 1.2 micromoles

Heart tissue homogenate equal to 0.112 and 0.224 mg/ml of nitrogen.

Acceptor system: glucose, 1.0 millimole
hexokinase (Sigma), 0.01 mg/0.2 ml.

TABLE XXIV.

DISAPPEARANCE OF PHOSPHOROUS IN THE PRESENCE OF 2-NITROPROPANE

2-nitropropane added (μmoles)	μmoles of 2-nitropropane per mg tissue N	μmoles P_i per mg tissue N	μmoles O_2 per mg tissue N	P/O
0.0	0.00	47.8	28.5	1.7
1.1	9.85	89.4	26.7	3.3
1.1	4.90	60.3	5.8	10.4
11.0	98.50	70.5	8.8	8.0
11.0	49.00	70.1	3.6	19.4
110.0	985.00	15.2	33.5	0.45
110.0	490.00	67.7	31.1	2.2

System:

Phosphate buffer, 0.15 millimoles, pH 7.4
Sodium succinate, 0.20 millimoles
Cytochrome c, 0.04 micromoles
$CaCl_2$, 1.2 micromoles
$AlCl_3$, 1.2 micromoles

Heart tissue homogenate equal to 0.112 and 0.224 mg/ml of nitrogen.

Acceptor system: glucose, 1.0 millimole
hexokinase (Sigma), 0.01 mg/0.2 ml.

2-nitropropane definitely increased the amount of inorganic phosphate consumed. This effect was evident over a 100-fold variation in the concentration of 2-nitropropane and was not a reflection of any effect of 2-nitropropane on the inorganic phosphate analysis. Likewise, examination of the effect of 2-nitropropane on the hexokinase system by the photometric method (Crane, K. and Sols, A. in S.P. Colowick and N.O. Kaplan editors, Methods in Enzymology, Vol. I, Academic Press, Inc., New York, 1957, p. 277), indicated that the observed increase in disappearance was not an effect on the acceptor system. The high P/O ratio resulted not only from a decreased oxygen consumption but also from the increased phosphate utilization.

In view of these observations and the chemical reactivity of 2-nitropropane the possibility of interaction with cytochrome c, DPN and DPNH was examined spectroscopically, under aerobic and anaerobic conditions. No change in spectra was observed for any of these compounds in the course of an hour at room temperature, in the presence of 2-nitropropane.

The possibility that the variations in the effects on the succinoxidase system might be reflection of the inhibition of cytochrome oxidase activity prompted examination of the oxidation of methylene blue under anaerobic conditions. In the presence of 2-nitropropane and heart tissue homogenate, reduced methylene blue was oxidized. The determinations shown in Figure 19 were made in closed 25 ml bottles stoppered with a sleeve-type rubber stopper. This procedure permitted simultaneous comparison of several tissue homogenate and 2-nitropropane concentrations. All materials were added under nitrogen and the 2-nitropropane was injected through the rubber stopper by means of a hypodermic syringe at zero time. Under these conditions the reduced methylene blue alone was not oxidized even after several hours,

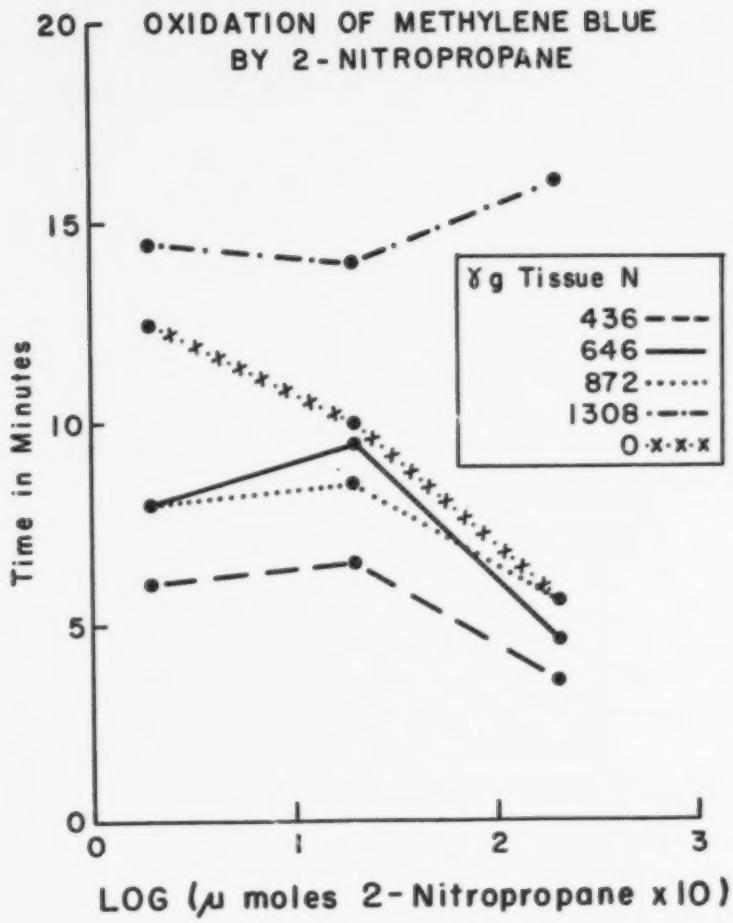

Figure 19. Oxidation of Methylene Blue by 2-nitropropane.

Methylene blue, 2×10^{-4} M (reduced).

2-nitropropane, 110.0, 11.0 and 1.10 μmoles added in 0.15 millimoles phosphate buffer, pH 7.4

Heart tissue homogenate nitrogen 2.18 mg/ml

Room temperature, nitrogen as the gas phase, total volume 3.0 ml.

hence it was assumed that the air was completely displaced. In the course of several hours it was possible to detect some oxidation of the methylene blue in the presence of the tissue homogenate alone.

The rates of oxidation of methylene blue in the presence of a constant concentration of tissue homogenate at various concentration of 2-nitropropane has been shown in Figure 20. The spectroscopic comparisons were made in a gas chamber cuvette constructed after a modification of the design of Lazarow and Cooperstein (Science, 129, 674, 1954). Since the apparatus provided for both gas inlet and outlet, nitrogen could be passed through the chamber again, without the complications of evacuation after the addition of the materials. With this apparatus the 2-nitropropane was introduced from a side arm. Under these conditions the reduced methylene blue was not oxidized by the 2-nitropropane at any concentration. This is in contrast to the observation shown in Figure 19 and suggests that only catalytic amounts of air are required for 2-nitropropane to oxidize reduced methylene blue. Here again, however, when all components of the system were present, the methylene blue was oxidized. Although not all concentrations of 2-nitropropane at all tissue concentrations included in Figure 19 have been reexamined, the indicated observations have been qualitatively substantiated as indicated in Figure 20.

As might be expected when the system is set up in the usual way and the reduction of methylene blue is followed in a nitrogen atmosphere, there was no observable reduction of methylene blue in the presence of 2-nitropropane. However, when triphenyltetrazolium chloride was used as a hydrogen acceptor the 2-nitropropane had no observable effect on the reaction, and the amount of triphenylformozan recovered was identical with that of the controls.

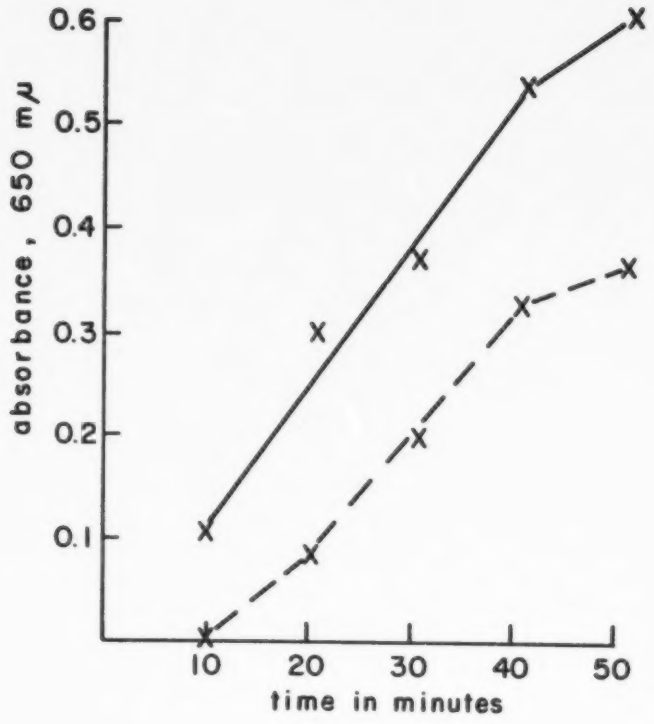

Figure 20. The Effect of 2-nitropropane on the Oxidation of Methylene Blue.

_____ in the presence of 110.0 μmoles 2-nitropropane

---------- in the presence of 11.0 μmoles 2-nitropropane

No change in absorbance in the absence of either the tissue nitrogen or the 2-nitropropane. System: phosphate buffer, 0.23 millimoles, pH 7.4
methylene blue, 1.6×10^{-1} micromoles
reduced with $Na_2S_2O_4$ and maintained under N_2
heart tissue nitrogen, 0.3 mg/ml
2-nitropropane in indicated concentrations
Nitrogen as the gas phase, total volume 3.0 ml

The Effect of the Pollutant Mixture on the Succinoxidase System and Phosphorylation.

Suspensions of guinea pig heart homogenate were directly exposed to the synthetic pollutant mixture in an exposure chamber shown in Figure 21. For these experiments dilutions of the heart homogenate of 1:20 and 1:30 were used. Typical results have been shown in Table XXV. For the 1:30 dilution the high P/O ratio results from decreased oxygen consumption. This is not true for the 1:20 dilution. These results are very comparable to those observed with 2-nitropropane.

Our method for determining aliphatic nitro compounds (Estes and Baughman, Anal. Chem. 33, 473, 1961) was modified for application to analysis of the pollutant mixture. However, there has been no indication that aliphatic nitro compounds as such are present in the pollutant mixture. The method is limited by the absorbant solution for trapping the gas and limits of concentration have not been established.

More important than the presence or absence of nitro compounds in the pollutant mixture was the previously mentioned similarity of effect of the pollutant mixture and of 2-nitropropane on the succinoxidase system and phosphorylation. The products of non-enzymatic oxidation of 2-nitropropane have been explored and there is evidence that the reaction proceeds via a free radical mechanism (Russell, J. Am. Chem. Soc. 76, 1595, 1954). These observations, in conjunction with the probability that formation of the pollutant mixture also involves a free radical mechanism, leads to the possibility that 2-nitropropane might serve as a model compound for studying such effects on enzyme systems and that further exploration of the effects of 2-nitropropane on the hydrogen transport system would be in order. Currently the effects of 2-nitropropane on mitochondria and

mitochondrial fragments are under investigation. After the characteristics of the preparations and the effects of the nitro compounds on them has been established, further comparisons with the effects of the pollutant mixtures can be made.

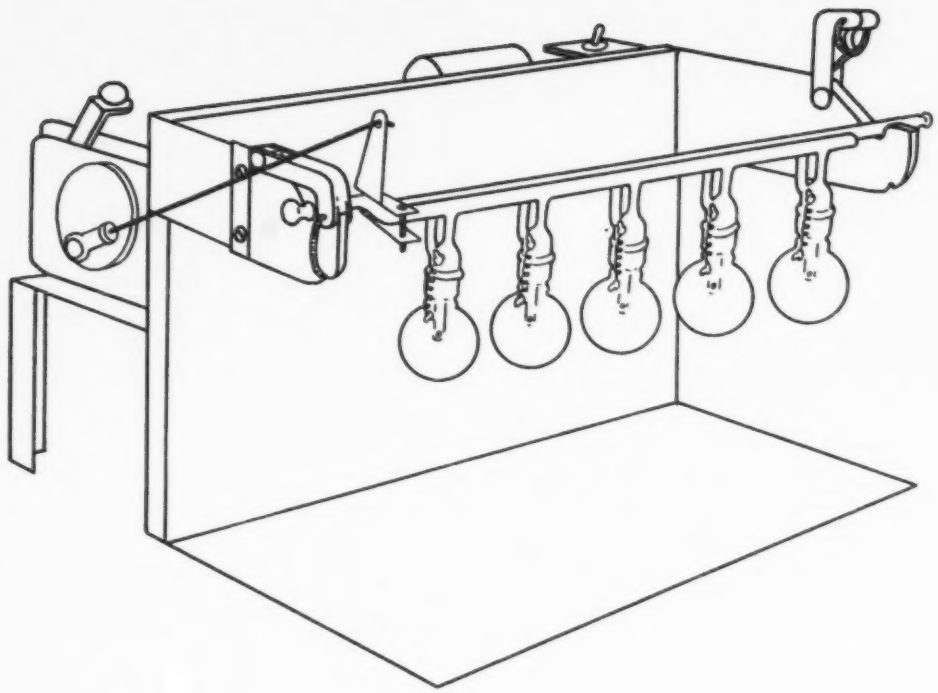

Figure 21

Table XXV

EFFECT OF DIRECT EXPOSURE OF HEART TISSUE HOMOGENATE TO SYNTHETIC AIR POLLUTANT MIXTURE

	1:20 dilution				1:30 dilution			
	N mg	O_2 µM/hr/mg	ΔPi µM/mg	P/O	N mg	O_2 µM/hr/mg	ΔPi µM/mg	P/O
Exposed smog	0.126	20.4	96.0	4.5	0.086	2.2	33.0	15.0
	0.251	17.8	122.0	6.8	0.172	0.4	146.0	36.5
Exposed air	0.126	35.6	273.0	7.7	0.086	30.6	100.0	3.2
	0.251	33.6	30.0	0.9	0.172	2.9	22.0	7.3
Unexposed	0.126	34.1	81.0	2.3	0.086	29.0		
	0.251	33.6	66.0	1.9	0.172	29.0		

Heart homogenate prepared in isotonic KCl, exposure time 15 minutes.
3.0 ml/vessel (exposure) of 1:20 or 1:30 dilution.
pH unchanged after exposure
Oxygen consumption and phosphorylation determined with succinoxidase system.

Analysis of the effluent:
Oxidant, µg O_3/l - 5.9
NO_2, ppm - 0.24
Acid, meq/l - 0.024
302 absorbing material as mg NO_3/l - 2.61

I. The Determination of Sulfur Compound in Tissues

The inhibition of aldolase and glutamic dehydrogenase activity by the pollutant mixture (this report) pointed to the probability that oxidation of sulfhydryl groups was involved in the biological effects of pollutant mixture.

In order to effectively investigate the role of sulfur and sulfur containing compounds, it became essential to determine the total sulfur content of the tissues. The divergence in results by previous methods suggested that complete oxidation of the sulfur components had not been achieved. The following method which involves oxidation of acetone dried tissues in a Parr Bomb Calorimeter and determination of sulfur by classic gravimetric procedures has proved to be simple, reliable and reproducible.

Methods for the determination of sulfhydryl and of disulfide groups is under investigation. With the development of these methods, the determination of the changes in the sulfur compounds of tissues resulting from exposure to the pollutant mixtures will be examined.

Method for the Determination of Total Sulfur in Tissues*

Procedure

Under ether anesthesia, animals were perfused with normal saline and the tissues excised. Acetone powders of the tissues were prepared by homogenizing in acetone and drying in the usual way.

The charge for the bomb was prepared by mixing 0.2 gram of acetone powder of the tissue with 0.3 grams benzoic acid, 1.00 grams of potassium perchlorate (accelerator) and 15 grams of sodium peroxide. All reagents were calorific grade. The mixture was subjected to combustion in a Parr Peroxide Bomb using a 7 cm. fuse of No. 34 B&S gauge resistance, and current of approximately 3 amperes at 9 volts.

The fusion mixture was quantitatively transferred to a vessel of hot distilled water and after dissolution, the sulfur was determined as barium sulfate by classic methods.

For the experiments herein reported, all tissues were obtained from male rats (Holtzman strain) of approximately 275 grams.

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* Presented 142nd meeting Am. Chem. Soc., Atlantic City, September, 1962.

Results and Discussion

The accuracy of the method was determined on triplicate samples of liver, kidney, spleen, lung, and small intestine. As shown in Table XXVI, the statistical evaluation showed that the mean standard error was not more than 0.04.

The method was found to be reproducible from animal to animal (Table XXVII), thus indicating that the preparation of the animals and samples was adequate and did not contribute an error in the determination. Attempts to compare the values for total sulfur content of tissues by this method with those reported in the literature has been shown in Table XXVIII. This comparison suggested that the method described herein leads to more nearly complete combustion of the tissue components and that the values so obtained more nearly represent the actual sulfur content of the tissues.

The method is applicable to micro-organisms or to physiological fluids such as urine and serum which have been freeze dried.

TABLE XXVI

REPRODUCIBILITY OF METHOD *

Sample No.	Liver	Kidney	Spleen	Lung	Intestine
1	3.80	2.18	3.69	3.68	1.84
2	3.85	2.23	3.61	3.61	1.82
3	3.76	2.16	3.73	3.65	1.80
Mean	3.80	2.19	3.67	3.65	1.82
Standard Error of Mean	0.03	0.02	0.04	0.02	0.01

*Dry weight basis

TABLE XXVII

PERCENT TOTAL SULFUR
(DRY WEIGHT BASIS)

Rat No.	Liver	Kidney	Spleen	Lung	Intestine
1	3.80	2.17	3.70	3.64	1.84
2	3.75	2.22	3.68	3.69	1.84
3	3.85	2.15	3.69	3.61	1.83
4	3.80	2.24	3.69	3.60	1.82
5	3.82	2.19	3.71	3.64	1.80
6	3.80	2.17	3.73	3.60	1.88
7	3.83	2.20	3.80	3.64	1.84
8	3.80	2.23	3.80	3.63	1.84
Mean	3.80	2.20	3.72	3.63	1.84
Standard Error of Mean	0.01	0.01	0.02	0.01	0.01

TABLE XXVIII

TOTAL SULFUR VALUES

Tissue	Dry Wt. of Acetone Powder-Grams	Av. Total Sulfur Mg/Gram	% Total Sulfur	Lit.*Values Mg./Gram
Liver	0.2	38	3.80	9
Kidney	0.2	22	2.20	
Spleen	0.2	37	3.70	
Lung	0.2	36	3.64	
(Small) Intestine	0.2	18	1.84	1.2

*Biochemists Handbook pp. 675, 780, Edited by Long, King and Sperry.

D. Van Nostrand Company, Inc., New York, 1961.

J. Determination of Kreb Cycle Components by Means of Gas Chromatography (in conjunction with RG-9695, formerly RG-6355)

Preliminary experiments have indicated effects of the pollutant mixtures on aerobic oxidation in tissues and point to the need for examining the utilization and production of Kreb Cycle components. These experiments have been limited by the methods of analyses available and the requirements for large volumes of samples. In exploring for a simple, sensitive and specific method for determining these compounds, gas chromatography of their methyl esters seemed most promising.

Trimethyl citrate and dimethyl succinate have been prepared by conventional methods, such as methanol-hydrochloric acid. These methods were not directly applicable for methyl lactate, pyruvate and fumarate. For the preparation of such esters, the diazomethane method presented the advantage of giving only nitrogen as a by-product, thus eliminating recovery problems frequently encountered.

Since most of the acids involved had not been previously examined for methylation by the diazomethane method, the question of the extent of methylation required consideration.

It has been reported that, with diazomethane, α-hydroxyl groups are methylated and that methyl pyruvate can further react with ultimate formation of ethylene acids. Furthermore, diazomethane can add to esters of unsaturated, conjugated, dibasic acids, e.g. fumaric, to form pyrazoline dicarboxylic esters, and on heating, the pyrazoline can decompose to form cyclopropane derivatives (B. Eistert "Syntheses with Diazomethane" in Newer Methods of Preparative

Organic Chemistry, Interscience, New York, 1948, p. 513).

In the investigations reported herein, the methyl esters of α-substituted and unsubstituted carboxylic acids were prepared by adding the free acid to an ethereal solution of diazomethane at room temperature. The method permits direct esterification of α-hydroxyl or keto substituted as well as α,β-unsaturated acids without side reactions. The methyl esters which were prepared, together with their properties, have been summarized in Table XXIX. The present increase in yield was achieved by recrystallization from ethanol.

For the esterification of α-keto acids such as pyruvic, the diazomethane method provided a simple means of obtaining a pure product in good yield.

This investigation led to the first preparation of the dimethyl ester of α-keto glutaric acid (2, 4-dinitrophenylhydrazone m.p. 120°C) and of the dimethyl ester of glutamic acid (ninhydrin and Hinsberg reaction positive).

For the separation and evaluation of the methyl esters by means of gas chromatography (Barbier-Colman Model 15 and 20), diethyleneglycol-succinate on Chromasorb P separated lactate, pyruvate, succinate and malate. For the columns thus far examined, citrate and fumarate are either retained or eluted so rapidly as to be under the solvent peak, or, as in the case of fumarate with an "Anabols" 3/4% NGS on Anakerom AB column, the retention time was the same as succinate. On all the columns, even at very low temperatures, α-ketoglutarate gave a single peak which coincided with that of methanol. The ease of decomposition of this compound suggests that it may be necessary to evaluate it in terms of the methanol formed. Further exploration of columns is in progress.

TABLE XXIX

SUMMARY OF DATA OF PREPARED ESTERS

PART 1

Liquid Esters	Dimethyl Succinate	Methyl Pyruvate	Dimethyl Malate	Methyl d, l-Lactate
B. P., Lit[a]	192.8°C.	137	242	144.8
This lab	106^{1} mm.	135.5	164^{1} mm.	144.2
η_D, Lit.[a]	$1.4197^{18°}$	$1.4046^{25°}$[b]	$1.4425^{20°}$	$1.4156^{16°}$
This Lab	$1.4120^{26°}$	$1.4108^{24°}$	$1.4404^{26°}$	$1.4124^{26°}$
d., Lit.[a]	$1.1202^{18°}$	$1.154^{0°}$	$1.223^{20°}$	$1.118^{0°}$
This Lab	$1.118^{25°}$	$1.148^{20°}$	$1.222^{22°}$	$1.051^{25.5°}$
Yield, Lit	33%[c]			
This Lab	86.4	41	73.7	69

TABLE XXIX

PART 2

Solid Esters	Trimethyl Citrate	Dimethyl Fumarate
M.P., Lit[a]	79°C.	102
This Lab	78	101
Yield, Lit	54%[c]	
This Lab	89.0	89.6

Unreported Esters	Dimethyl Glutamate	Dimethyl α-Ketoglutarate
M.P.		120°C. (2, 4-dinitrophenyl-hydrazone)
η_D	$1.3919^{26°}$	$1.4003^{26°}$
d.	$1.059^{25°}$	$1.109^{25°}$
Yield	71.2%	69.5

a. Handbook of Chemistry and Physics, 40th Ed., Chemical Rubber Publ. Co., Cleveland, Ohio, 1959.

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c. R. Roper and T. S. Ma, Microchemical Journal 1, 245 (1957).

INFORMATION DEVELOPED UNDER THIS GRANT HAS BEEN PRESENTED IN THE FOLLOWING

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PROFESSIONAL PERSONNEL EMPLOYED

Research Associates: Chao-Han Pan* (Jan.-July, 1960; Aug., 1962 -)
Alma W. Coats** (Sept., 1961 -)

Technicians: Nancy S. Austin** (Jan., 1960 - June, 1961)
Pauline K. Baughman* (Jan., 1960 - June, 1961)
Robert McGivney* (May, 1960 - March, 1961)
Robert C. Duncan* (July, 1962 -)

* Denotes full time.

** Denotes half-time.

APPENDIX A

AN IMPROVED APPARATUS FOR THE PREPARATION OF AIR POLLUTION MIXTURES

With the previously described apparatus for the production of simulated air pollution mixtures (this report p. 1) variations in the biological effects were observed which were not reflected in chemical analyses (appendix B). Evaluation of this observation led to reexamination of the system for production of the pollutant mixture.

Although not reflected in the usual chemical analyses, samples of the effluent taken at varying times after the injection of the hydrocarbon gave variable patterns of analyses by gas chromatography. The variations were less marked as smaller amounts of hydrocarbon were injected at more frequent intervals. This suggested that a cyclic series of reactions was occurring which varied with the concentration of hydrocarbon present.

Likewise, it was observed that after repeated irradiation of the pyrex reaction flasks, the effluent showed less biological activity and, in some cases, a decrease in the apparent concentration of the chemical components.

Elimination of these variables in the production system was essential to the further exploration of the biological observations as well as any comparison of biochemical effects and chemical determinations.

In the redesigned apparatus the hydrocarbon was metered into a 5 liter flask along with air such as to provide a preliminary dilution of the hydrocarbon. To further aid in the mixing of the gases previous to irradiation and to provide for further dilution, a 5 liter premix flask was used. Dilution to the desired

hydrocarbon concentration was achieved by splitting the gas stream from the preliminary dilution flask, rejecting the larger portion, and sweeping the trickle flow into the premix flask with an adjustable stream of air. A similar dilution system provided for continuous flow of the NO_2 into the premix flask. (Shown in Figure A-1) Control of the flow of the gas streams was achieved by the use of 1-1/4 mm. glass needle valves (Fisher Porter No. 795-500-0114).

From the premix flask the gas mixture was led directly into the reaction flask. To eliminate the variation in irradiation, a quartz, high pressure 450 W mercury vapor lamp (Hanovia Lamp Div., Engelhard Industries, Inc.) was immersed directly into the reaction flask. For temperature control, the lamp was fitted with a sleeve condenser permitting the continuous flow of water. Irradiation systems of this type have frequently been used for the study of organic reactions.

The lamp housing was fitted into the center opening of a three necked flask. The other two openings were fitted with inlet and outlet tubes. In the initial design it was assumed that with the more efficient irradiation system a 5 liter flask could be used for the reaction and still permit a flow of 200 to 600 ml per minute. Addition of plug (insert in the drawing) and bubblers permitted higher tank pressure at lower flow rate, thus stabilizing the input system.

Other than the irradiation unit, all the glass was pyrex; the flasks were fitted with S joints, and connecting lines were of 9 mm. tubing fitted with ball and socket joints, as were the connections to the glass flowmeters.

A 3

Preliminary test of the apparatus included only chemical determination. The hydrocarbon concentrations were determined on a Barber-Colman Model 20 Gas Chromatograph with a squalene capillary column at 75° C and nitrogen oxides by the Saltzman method. Total oxidants were determined by the phenolphthalin method, and ozone by the diphenylamine sulfonate (NaDS) method (Bovee and Robinson, Anal. Chem. 33, 1115, 1961). The ultra-violet absorbance of a 4% sodium hydroxide solution exposed to the effluent mixture was also examined.

Tests of the apparatus at a flow rate of 240 ml/min. with air containing only butane indicated a progressive increase in the butane peak by chromatographic analysis. The entire system reached a steady state in 7 hours after which there was no change in the hydrocarbon peaks of the unirradiated system. On removal of the hydrocarbon from the system the butane concentration decreased at a rate comparable ($3.0 \pm 1.0\%$) to the previous increase in concentration. These observations were interpreted as indicating that good mixing had been achieved. Further studies showed that the apparent "mean residence time" in the reaction flask was 50 min.

Our observations confirmed those of Saltzman (Anal. Chem. 26 1949, 1954) that the use of 10% NO_2 in air avoided considerable trouble. For the initial evaluation of the system, however, undiluted nitrogen dioxide was used. This required operating close to the lower limit of the NO_2 splitting system and a more critical test for the control of concentrations of input gases. The variation of NO_2 concentration is seen in the analysis shown in Figure A-2 and A-3. For these experiments the NO_2 input was 4 ppm from

flow calculations and 3.4 ppm by analyses. From these data it is evident that from 3 to 7 hours before irradiation there was less variation in the analyses by the Saltzman test, and therefore in the NO_2 concentration, then in the phenolphthalin reaction. The latter variation was not reflected in the NaDS analysis for ozone.

The experiments shown in Figure A-2 and A-3 differ only in the hydrocarbon used. For each experiment, after irradiation, the NO_2 concentration dropped to comparable values. (0.6-0.7 ppm) For the butane system there was little change in the phenolphthalin test for total oxidant concentration before and after irradiation even though the NaDS reaction indicated no ozone formation. These observations suggested that before irradiation the response of the phenolphthalin reaction was to NO_2 while after irradiation it was primarily to ozone. Not shown in the figure was the observation that the butane peak of the chromatographic pattern was unchanged in height, area or shape throughout the experiment.

For the butene system, after about one hour of irradiation the butene peak was reduced by 1/3 with no evidence of trailing in the chromatographic pattern. After two hours it was apparent that the butene peak was no longer symetrical and two other components with retention timing 3 and 18 seconds after butene (retention time, 80 sec.) appeared.

The marked increase in total oxidants and the slow increase in ozone for the butene as compared to the butane system indicated that with butene the oxidants formed were not ozone, nor increased NO_2 but possibly products

of NO_2 and butene. Whether such products are represented in the two new chromatographic peaks has yet to be determined.

Initially it was hoped that the NaDS method could be used both for ozone (590 mμ) and NO_2 (410 mμ). The determination of the absorbance at 410 mμ, however, was too rapid for dependability. This has been shown in Figure A-4. The time ordinate represents time from removal of the sample to reading.

With the butane system the 410 mμ absorbance did not indicate a decrease in NO_2 concentration comparable to that shown by the Saltzman reaction. This observations was not what would be expected from the data of Figure 4. The observations suggested the possibility that by irradiation of NO_2 and butane, products were being formed which on gas chromatography appeared with the butane peak but which gave back NO_2 to the NaDS reaction.

With this system, after irradiation of the gas mixture the previously unidentified ultra-violet obserbing (300 mμ) material collected in basic solution was no longer evident. The irradiation did, however, produce material which absorbed in the 225-230 mμ region.

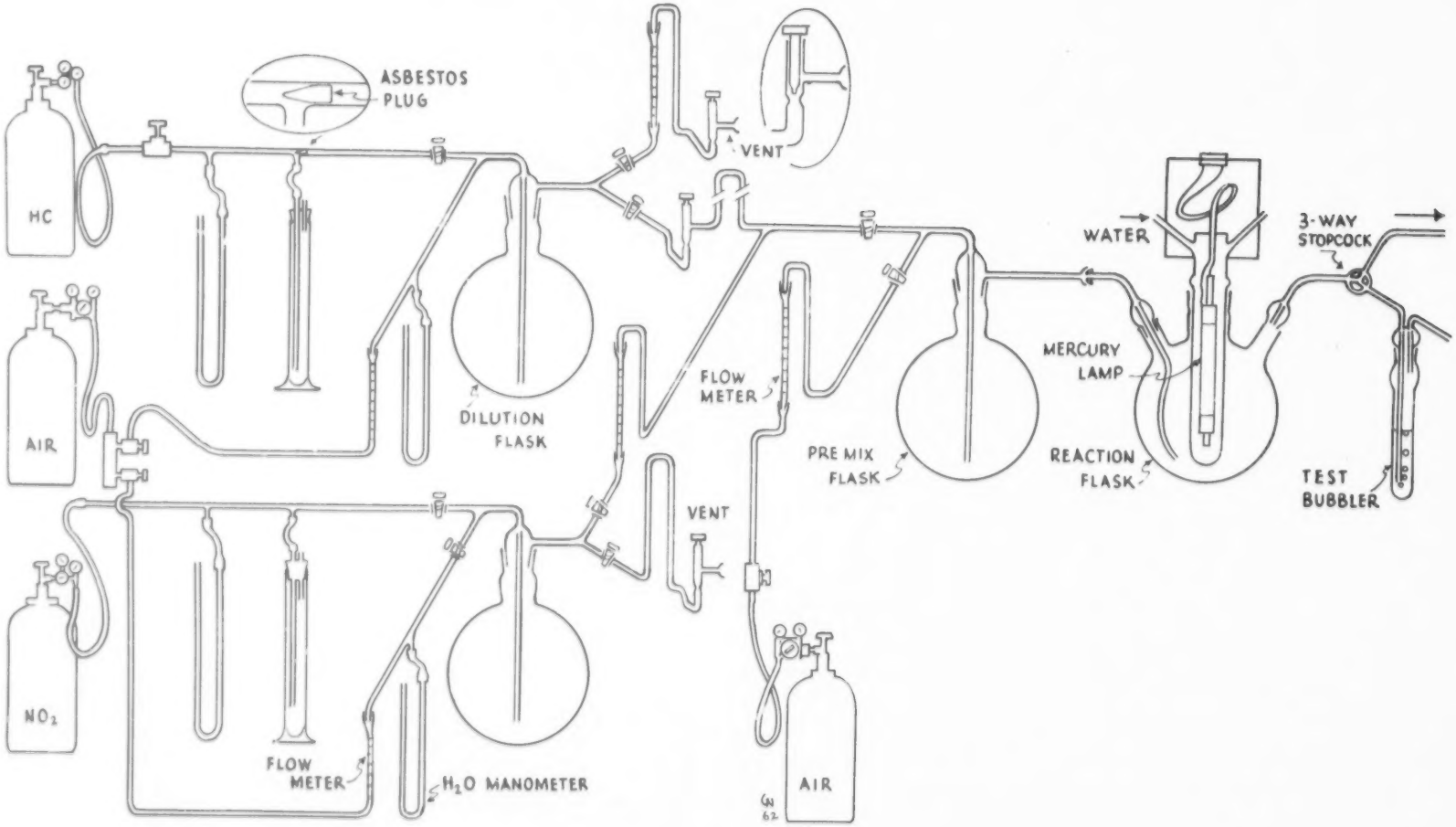
Solutions of sodium nitrate in concentrations of 0.05 mg/ml and below show no absorbance in the 300 mμ region but strongly absorb in the 225-230 region. Nitrites as well as nitrate absorb in the latter region. It is possible that irradiation leads to the formation of either or both of these ions.

The disappearance of the 300 mμ absorbance is consistant with our previous observation that aliphatic nitro compounds could not be detected in the pollutant mixture by chemical means (Estes and Baughman, Anal.

Chem. 33, 473, 1961). Although the limitations of the chemical determination did not completely eliminate the possible presence of the aliphatic nitro compounds in the effluent, the disappearance of the 300 mμ absorbance when compared with previous data (Gast and Estes, Anal. Chem. 32 1712, 1960) suggested that, if present, the concentration would be less than 1 micromole per liter for nitromethane and nitroethane.

It has been tentatively concluded that the previously observed 300 mμ absorbance resulted from nitrate ion entrained or otherwise carried over when nitrogen oxides were obtained by passing air through nitric acid.

The initial criterion of biological effectiveness of the effluent gas has been the inhibition of growth of E. Coli following exposure to the pollutant mixture (see p. 7). With butene as the sole source of hydrocarbon the increased lag time for the subsequent growth of E. Coli was marked after 15 minutes and prolonged after 30 minute exposure. Under these conditions no ozone formation was detected. Likewise, at various input concentrations the NO_2 concentration in the irradiated effluent gas was consistantly less than 0.7 ppm. Since 1 ppm of NO_2 does not produce observable lag time for E. Coli for exposure times of less than 1 hour, it is eliminated as an effective agent per se for this biological system.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PLUG
VENT
HC
WATER
3-WAY
STOPCOCK
AIR
DILUTION
FLASK
FLOW
METER
MERCURY
LAMP
PRE MIX
FLASK
REACTION
FLASK
TEST
BUBBLER
VENT
NO_2
FLOW
METER
H_2O MANOMETER
AIR
GN
62

Figure 1-A

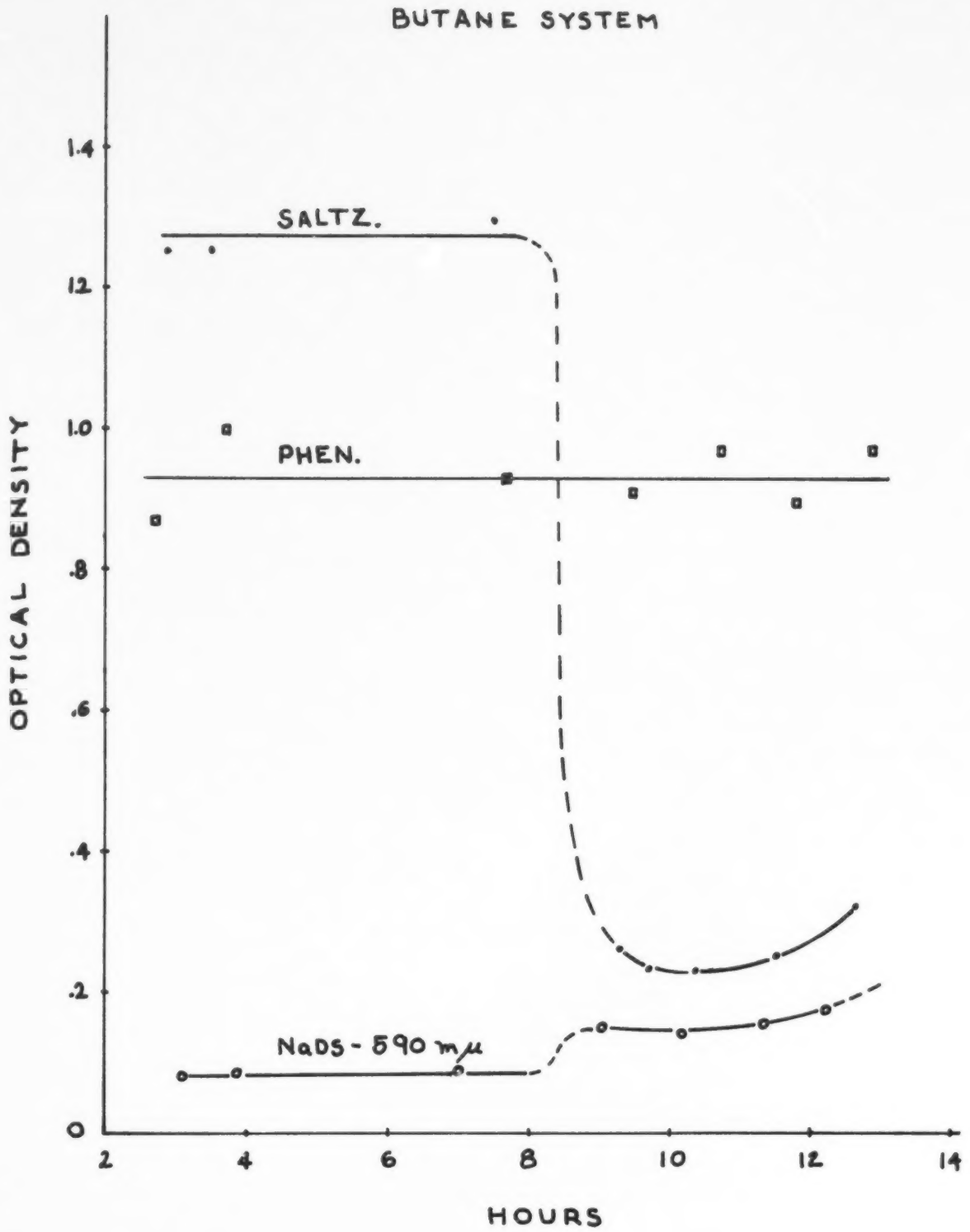
BUTANE SYSTEM
SALTZ.
PHEN.
NaDS - 590 mμ
OPTICAL DENSITY
1.4
1.2
1.0
.8
.6
.4
.2
0
2
4
6
8
10
12
14
HOURS

Figure 2-A

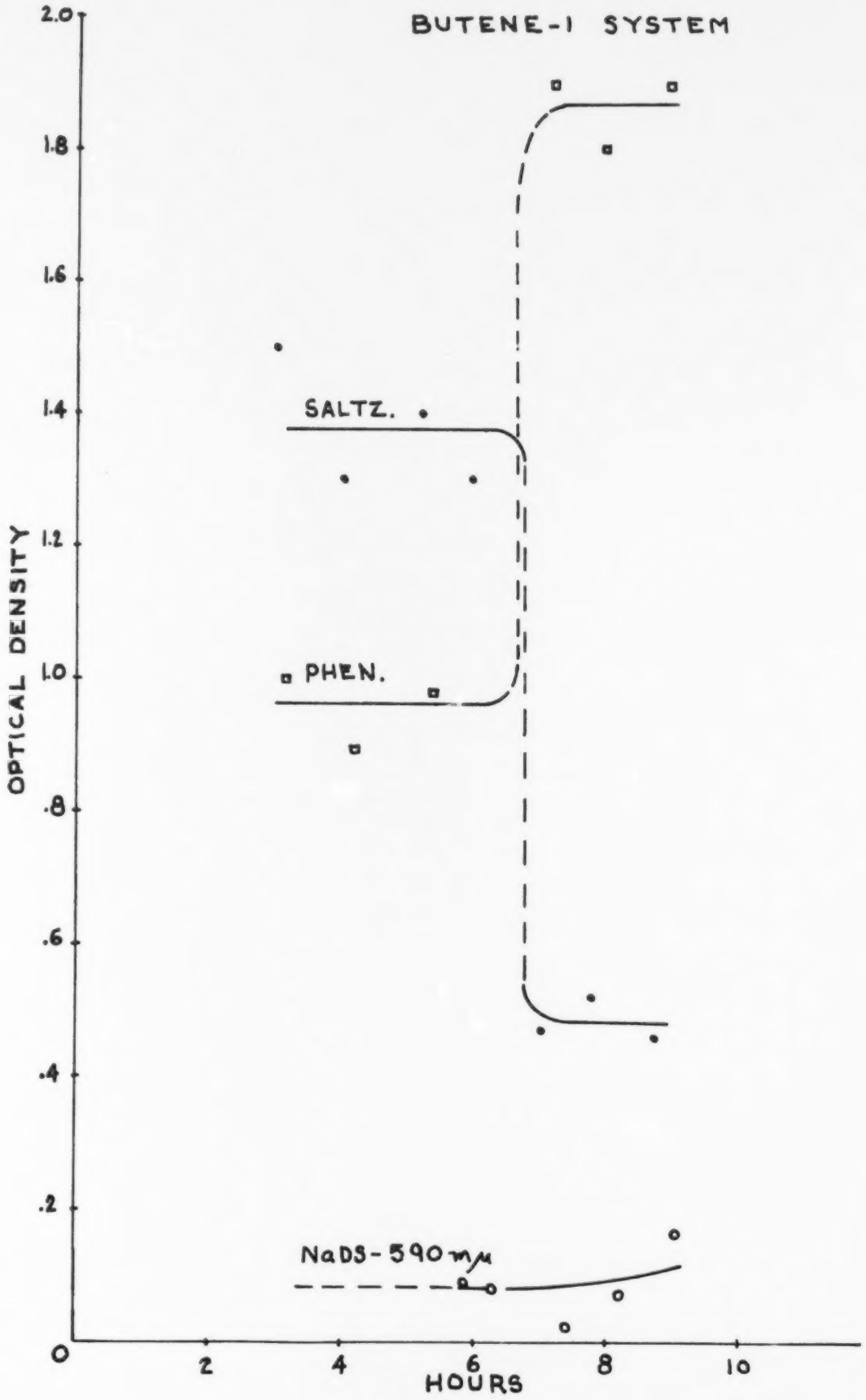
BUTENE-1 SYSTEM
OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
SALTZ.
PHEN.
NaDS-590 mμ
2
4
6
8
10
HOURS

Figure 3-A

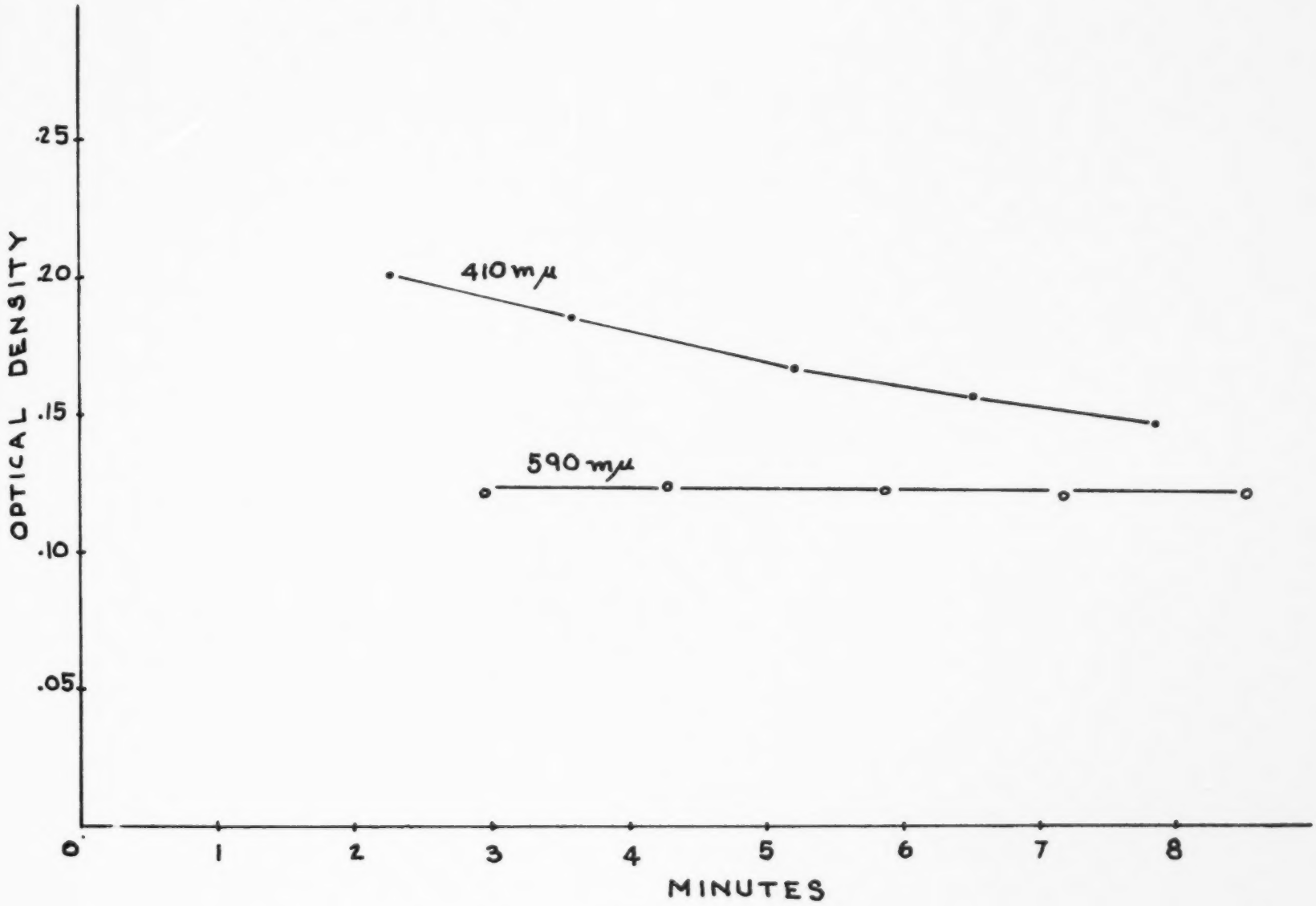
OPTICAL DENSITY
.25
.20
.15
.10
.05
0
1
2
3
4
5
6
7
8
MINUTES
410 mμ
590 mμ

Figure 4-A

APPENDIX B

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Analysis of Air Pollution Mixtures: A Study of Biologically Effective Components

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▶ **The biological effectiveness of an oxidant-type air pollution mixture was determined from the inhibition in growth of E. coli following exposure to the pollution mixture. Absorption in phosphate buffer and adsorption on a column of C-22 Firebrick coated with 20% (w./w.) disodium phosphate appeared to remove completely the effective components. Both agents removed a part of the oxidant material as determined by the phenolphthalin method. The material absorbed in the phosphate buffer markedly inhibited the oxygen consumption of leucocytes exposed to it. The buffer completely removed the as yet unidentified 302-mμ absorbing material. However, the apparent concentration in the buffer did not correspond to the apparent concentration in the gas. The data suggested that the 302-mμ absorbing material contained a biologically effective agent, which might be peroxyacetyl nitrate.**

EXTENSIVE studies have been made on the analysis of air pollutant mixtures and automobile exhaust fumes. Air pollutant mixtures have been shown to damage plants and bacteria. The voluminous literature on the analysis of the pollutant mixture indicates the complexity of the mixture and some of the problems encountered in chemical analyses.

In an effort to examine specific biochemical effects of a pollutant mixture, the question of analysis becomes a most pressing one. Are the observed biochemical effects evoked by a single or multiple component(s) of the mixture? Assuming that the effective agent is a single component, the probability remains that other components have a synergistic effect on the biochemical system or are themselves converted to more reactive compounds. These possibilities are emphasized by the requirement of irradiation for the formation of pollutant mixture. To explore the in vitro biochemical effects of an oxidant-type air pollution mixture, two general problems are involved:

Determination of Specific Effects. Because of the nature of biological systems, direct effect on the system must be distinguished from effects on the substrate or environment of the system, or on the method of determination.

Determination of Specific Cause. Since we are dealing with a complex mixture, direct evidence for indicating particular biochemically reactive agents requires either separation of these agents from the rest of the mixture or the ultimate in specificity from the method of analysis.

Inherent in each of these is a third problem, namely, the possibility that the means of separation as well as the method of determination can of themselves be involved in the cause and in the effect.

Our previous work (*13*) and that of Goetz and Tsuneishi (*8*, *9*) showed that an oxidant-type air pollution mixture inhibited the rate of subsequent growth of *E. coli*. The development of equipment for uniform exposure of cells and the measurement of subsequent growth led to a reproducible method for determining inhibition (*13*). For known compounds, an index of effectiveness of gaseous agents on microorganisms involving concentration of the agent and time was indicated (*13*). For the pollutant mixture the use of such an index was not feasible since the particular inhibitory compound(s) had not been identified.

Further examination of the effects of the pollutant mixture on *E. coli* indicated that glutamic dehydrogenase was probably one of the enzymes involved (*4*). The characteristics of this enzyme (*12*) suggested that the inhibition resulted from oxidation of the sulfhydryl groups. This hypothesis could not be directly demonstrated since, using either per cent bacteria killed or increase in lag time as an index of biological effect, there was no simple relationship between the biological effect and the usual chemical analysis of the pollutant mixture for oxidants.

Methods for determining oxidants are not specific; the phenolphthalin method, for example, includes as oxidants ozone, nitrogen oxides, alkyl nitrites, peroxyalkylnitrites, and a variety of peroxides (*10*). Since there is no specific method for differentiation of the oxidants, separation of components in the pollution mixture was attempted. Three general approaches to separation of the gas mixture were used: cold trapping, absorption in liquids, and adsorption on solids.

PROCEDURE

The general criterion of biological effectiveness used was the inhibition in growth of *E. coli* following exposure to the pollutant mixture or fraction. The apparatus and techniques required have been described in detail (*13*). In brief, a thin layer of organisms, collected on a Millipore filter, was exposed to the gas stream, transferred to a growth chamber, and the degree of inhibition was determined as a function of time, from the change in absorbance of the medium.

The aldolase studies were made on cell-free extracts of *E. coli* prepared from a 24-hour broth culture. The washed cells were suspended in sterile water and the cells were ruptured in a sonic oscillator. The ruptured cell suspension was centrifuged at 25,000 × g for 30 minutes at 0° C., and the supernatant

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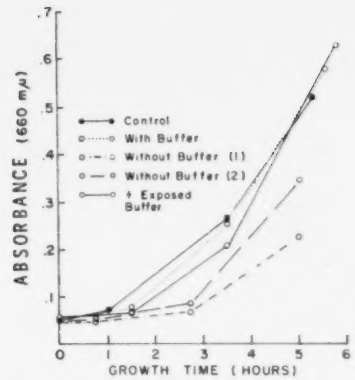

Figure 2. Effect of the pollutant mixture, after bubbling through phosphate buffer, on growth of *E. coli*. Exposure time 60 minutes

Analysis of line:
Oxidant, μg. O_3/l., 6.6
NO_2, p.p.m./l., 0.23
Acid, meq./l., 0.025
302 mμ as mg. NO_3/l., 3.06

removing the as yet unidentified 302-mμ absorbing material (see analytical section).

To determine the concentration of these materials in the absorbing solutions, the method of analysis for oxidant was modified, since the presence of phosphate interfered. Sodium chloride solution of comparable ionic strength and pH was substituted. Comparison of the analysis of the gas and the solution is given in Table IV. In all experiments the analysis of the absorbing solution indicated higher oxidant concentrations than in direct analysis of the gas. Does this mean that there was potential oxidant material in the gas which became actual oxidant material in solution?

In exploring column packing materials for gas chromatographic analysis, we observed that for a variety of columns, mineral acids could be quantitatively eluted from the columns and that the retention properties of the column appeared to change gradually when acid-containing samples were analyzed. This observation led to exploring suitable column packing materials which would effectively remove acids from the gas samples without removing other polar compounds which might be present. Studies on C-22 Firebrick, 30- to 60-mesh, coated with 20% (w./w.) disodium phosphate packed in 5-cm. columns (7-mm. i.d.) indicated quantitative removal from an air stream of organic, as well as inorganic, acids as represented by hydrochloric, phosphoric, nitric, acetic, propionic, and butyric acids.

The disodium phosphate–Firebrick column also permitted only semiquantitative recovery of representative alcohols, ketones, and nitro-hydrocarbons having boiling points of 132° C. or less. Thus, each of these compounds is held in part by the column.

With a column of 20% (w./w.) disodium phosphate on Firebrick in the effluent line, good recovery of the growth rate was exhibited by the organisms exposed to the pollutant mixture. As seen in Figure 3 the growth rate of the exposed organisms

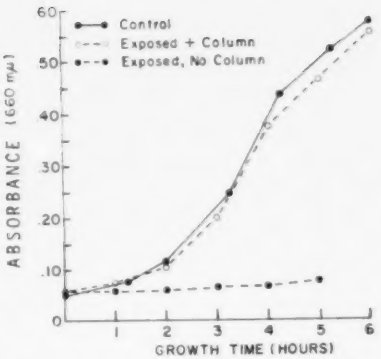

Figure 3. The protective effect of a disodium phosphate—Firebrick column on the growth of *E. coli*

Analysis of the line:

	Column Present	Column Absent
Oxidant, μg. O_3/l.	6.6	1.0
NO_2, p.p.m.	0.31	0.59
Acid, meq./l.	0.029	0.001

Column: 5 cm. of 7 mm. i.d. tubing containing C-22 firebrick, 30- to 60-mesh coated with 20% (w./w.) Na_2HPO_4

was identical with that of the unexposed control.

From the analysis of the effluent before and after the column, it would appear that oxidants were the most significant components removed in terms of quantity. Further comparisons of the analyses of the pollutant mixture behind these columns are shown in Table V. These experiments represent different columns of the same dimensions and packing. Of the components tested, the nitrogen dioxide appeared to be the most variable. Attempts to elute possibly absorbed materials from the column were unsuccessful.

Exposure of purified glutamic dehydrogenase to the pollutant mixture led to difficulties in the determination of enzymatic activity. Aldolase from *E. coli* is also regarded as a sulfhydryl enzyme (*1*). Examination of the effectiveness of a disodium phosphate–Firebrick column for the protection of aldolase activity has shown that the column absorbed acid materials (Table VI). Were this the only characteristic of these columns, it might be assumed that the more effective inhibitor of aldolase activity was a decrease of pH. However, decreasing the pH alone, as with HCl, gives complete inhibition with recovery of activity after pH adjustment. This does not simulate the effect of exposure to the pollutant mixture. The data indicate that the pollutant mixture has a twofold effect on aldolase activity. In addition, the magnitude of the increased activity for aldolase exposed behind the column cannot be ignored. This apparent stimulation by the column requires further investigation.

Table III. Effect of Bubbling Pollutant Mixture through Phosphate Buffer

	Oxidant, μg. O_3/Liter of Gas	NO_2, P.P.M.	Acid, Meq./Liter	302 Mμ, as Mg. NO_3^-/Liter
Direct	6.3	0.33	0.028	2.35
After phosphate buffer	4.6	0.12	0.002	0.00

Table IV. Concentration of Pollutant Materials in Gas and Absorbing Solution

Analysis of the Gas				Analysis of the Solution Calculated to Gas	
O_3, μg./liter	NO_2, p.p.m.	Acid, meq./liter	302 mμ as mg. NO_3/liter	O_3, μg./liter of gas	302 mμ as mg. NO_3/liter of gas
4.9	0.33	0.028	2.83	6.1	1.75
5.4	0.51	0.030	2.57	7.5	1.99
5.3	0.36	0.028	2.52	6.3	1.75
5.2	0.36	0.036	2.89	9.1	2.46
4.7	0.38	0.028	2.78	10.5	2.69

Table V. Analysis of Pollutant Mixture after a Disodium Phosphate Column

	Oxidant, μg. O_3/Liter	NO_2, P.P.M.	Acid, Meq./Liter	302 Mμ as Mg. NO_3/Liter
Without column	6.9	0.28	0.029	2.27
With column	0.2	0.13	0.004	0.13
Without column	6.5	0.26	0.028	1.91
With column	1.2	0.25	0.007	0.89

From the data it was apparent that glass wool can partially remove the biologically effective components.

The phosphate buffer and the disodium phosphate–Firebrick column appeared to remove completely the effective components. Both trapping agents removed a part of the oxidant material. It was not apparent whether the remaining oxidant material was biologically ineffective or in too low a concentration to be detected. Likewise current methods would not permit differentiation among oxidant materials.

The observation that the biologically effective material was water soluble eliminated the possibility that ozone was the sole offending component. Although considerable evidence points to the destruction of ozone by surface action (*14*), ozone has only slight solubility in water.

It was observed that the 302-mμ absorbing material was completely removed by the buffer and that the solution did not account for the concentration of the absorbing material in the gas. Furthermore, the apparent oxidant material had increased in concentration. These observations suggested that the 302-mμ absorbing material contained a biologically effective nitrogen containing agent apparently not accounted for in the nitrogen oxides as determined by the Saltzman method.

The peroxyacetyl nitrite, suggested by Scott *et al.* (*16*), is less likely a component of the 302-mμ absorbing material than the recently suggested peroxyacetyl nitrate (*18*). Although nitromethane is a product of the slow decomposition of the peroxyacetyl compound, no evidence of nitro compounds was found in the effluent. The limitations of this method (*2*) in determining nitromethane do not completely eliminate its presence in the gas.

Further examinations of the analysis of the pollutant mixture and exploration of its biochemical effects are in progress.

ACKNOWLEDGMENT

The author appreciates the excellent technical assistance of Chao-Han Pan, John A. Cooper, and Pauline K. Baughman.

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Table VI. Removal of Aldolase Inhibition by Disodium Phosphate–Firebrick Column

	pH	Aldolase Activity, Mμmole/Ml./Hr.
Unexposed	7.5	0.63
Unexposed, acidified	4.0	0.00
Exposed without column	4.0	0.15
Adjusted	7.5	0.36
Exposed with column	7.5	0.92

Column
5-cm. column of 7 mm. i.d. tubing containing C-22 Firebrick, 30- to 60-mesh, coated with 20% (w./w.) Na_2HPO_4
Analysis of line
Oxidant (μg. O_3/liter), 6.1
NO_2 (p.p.m. NO_2/liter), 0.38
Acid (meq./liter), 0.027
302-mμ absorbing material (mg. NO_3/liter), 2.12

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